5' heterogeneity of glucocorticoid receptor mRNA: associations with tissue-specific, constitutive and auto-regulation

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Declaration

I declare that this thesis and the work presented in it are entirely the result of my own independent investigation, except where stated in the text. This work has not been, and is not currently, submitted for any other degree.

Helen L. Munn

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5-HT	5-hydroxytryptamine
5'-RACE	5' rapid amplification of cDNA ends
11βHSD1/2	11β-hydroxysteroid dehydrogenase type 1/2
ACTH	Acetyltransferase nuclear receptor coactivator
AP1/2	Activator protein ½
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
bp	Base pairs
BST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding protein
cDNA	Complementary DNA
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
Dex	Dexamethasone
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Egr-1	Early growth response gene 1 (a.k.a. NGFI-A)
EMSA	Electrophoretic mobility shift assay
ER	Oestrogen receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HAT	Histone acetyltransferase

HPA	Hypothalamic-pituitary-adrenal
HS	Hypersensitive
Hsp	Heat shock protein
IAA	Isoamyl alcohol
IκB	Inhibitory κB
IL-1/2/6	Interleukins 1/2/6
kb	Kilobase
LBD	Ligand binding domain
MeCP	Methyl CpG binding protein
MMTV (LTR)	Mouse mammary tumour virus (long terminal repeat)
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger RNA
MR	Mineralocorticoid receptor
nGRE	Negative GRE
NF-1	Nuclear factor 1
NF-κB	Nuclear factor κB
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PMSF	Phenylmethylsulfonyl fluoride
PNMT	Phenyethanolamine-N-methyl-transferase
POMC	Pro-pionomelanocortin
PR	Progesterone receptor
RNA	Ribonucleic acid
RPA	Ribonuclease protection analysis
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
sORF	Short open reading frame
Sp1	Selective promoter factor 1
SSC	Saline sodium citrate

STAT	Signal transducer and activator of transcription
SWI-SNF	Switch-sucrose-non-fermentable
TAT	Tyrosine aminotransferase
TBE	TRIS boric acid EDTA
TBP	TATA binding protein
TCR	T cell receptor
TEMED	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
TFIID	Transcription factor II D
YAC	Yeast artificial chromosome

Publications from this thesis

Papers

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Abstracts

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Abstract

Glucocorticoids are steroid hormones that exert profound effects on virtually all tissues. The principal determinant of cellular sensitivity to glucocorticoids is the level of glucocorticoid receptor (GR). Despite near-ubiquitous expression, GR levels vary widely between tissues. A complete lack of GR is lethal at birth and even mild dysregulation has significant pathophysiological consequences. The rat GR gene includes a complex 5' promoter region encoding at least eleven untranslated alternate exons 1, eight of which lie in a ~3kb CpG island region close to exon 2. The subject for this investigation has been the association between the alternate exons 1 of the GR promoter and the tissue-specific, constitutive and auto-regulation of GR.

Cell-specific alternate exon 1 expression was analysed by RT-PCR in various rodent cell lines. All 'CpG island' exons 1 examined (1_4, 1_5, 1_6, 1_7, 1_{10} and1_{11}) were expressed in all cell lines tested, while expression of exon 1_1, which lies outside the CpG island region, ~30.2kb upstream of exon 2, was restricted to cells of the immune system. Promoter activity was analysed by transient transfection in B103 (rat neural), C6 (rat glioma) and H4IIE (rat liver) cell lines, using constructs forming both a 3' and 5' deletion series of the CpG island region fused to a luciferase reporter gene. 'P2' contains the entire promoter and was the most active construct in all cell lines tested. Transfections using the 3' deletion series, which assesses individual exon 1-associated promoter activity, demonstrated that only '$P1_7$' showed high tissue-specific activity restricted to the neural cell lines B103 and C6. Further transfections with constructs based on $P1_7$ identified a 134bp region required for high promoter activity in neural cells, which was orientation-specific and did not show properties associated with a classical enhancer. This directionality was mirrored in *DNaseI* protection studies in which a footprint was identified on only one strand of the 134bp fragment. While gel mobility shift assays showed evidence of Sp1 binding over the region, the physiological significance of this remains unclear. It may be the case that this fragment represents a region at which directional transcription is initiated. Transfections in B103 cells using the 5' deletion series identified two regions important for high promoter activity. Further analysis showed that the

activity of these regions was position-dependent within the context of the whole promoter. It appears, therefore, that alternate GR exons 1 are not associated with individual promoters that are selectively regulated in different cell types. Rather, tissue-specific levels of GR arise through a complex arrangement of interacting regulatory elements over the whole promoter region.

The role of individual exon 1 splice donor sites was investigated by site-directed mutagenesis of splice donor sites at exons 1_6, 1_{10} and 1_{11} within P2. Following transient transfection in B103 cells, none of the mutant constructs showed a loss in promoter activity relative to intact P2. RT-PCR identified longer transcripts associated with the mutant exons, reflecting the use of downstream 'cryptic' splice donor sites. There appears to be redundancy between alternate exons 1, which can therefore be seen to form a robust promoter mechanism that ensures constitutive GR expression. *DNaseI* hypersensitive site mapping in H4IIE cells showed that a ~3kb fragment, encompassing the entire CpG island region, is bounded by hypersensitive sites and shows general sensitivity to nuclease digestion, consistent with an extended region of transcriptionally active chromatin.

The role of individual exons 1 in the autoregulation of GR was investigated by transient transfection of the 3' and 5' deletion series of the GR promoter into B103 cells treated with the synthetic glucocorticoid, dexamethasone. Results showed that the mechanism of autoregulation does not operate selectively on individual exons 1, but instead down-regulates the transcriptional activity of the whole GR CpG island region.

Results were therefore consistent with a model of GR transcriptional regulation in which exons 1 are not associated with individual promoters that are selectively regulated by tissue-specific or auto-regulatory signals; rather, regulation occurs through position-dependent interactions of elements arranged over the whole CpG island promoter 'region'. However, while selection of alternate exon 1 transcription initiation sites may not be of primary importance to the tissue-specific or auto-regulation of GR, the multiple exon 1 structure does ensure that promoter activity is

especially robust to mutations and thus ensures the constitutive expression of this vital housekeeping gene.

1 Introduction

1.1 Glucocorticoids

Glucocorticoids are steroid hormones that are secreted by the adrenal glands and are known to regulate a large and varied range of physiological processes. They are principally involved in restoring physiological homeostasis after stress, but also play key roles in intermediary metabolism, development, central nervous system function and the immune response. Dysregulation of circulating glucocorticoid levels is associated with a number of pathologies, most notably Addison's disease and Cushing's syndrome. In cases of the former, primary adrenal insufficiency leads to deficient glucocorticoid production which is characterised by weakness, fatigue, weight loss and gastrointestinal complaints (Orth et al., 1992). In the latter, excessive glucocorticoid secretion causes a range of symptoms including central obesity, muscle atrophy, hypertension, diabetes, osteoporosis, depression and memory loss (Orth et al., 1992).

Glucocorticoids, like all steroid hormones, exert their effects on target cells by interacting with intracellular receptor proteins. Two types of receptors are known to bind to glucocorticoids; the mineralocorticoid receptor (MR), which binds ligand with high affinity and the glucocorticoid receptor (GR), which binds with lower affinity (Orth et al., 1992). Both receptors are members of the ligand-activated transcription factor super-family and therefore regulate a large number of target genes by a variety of mechanisms (reviewed in Beato et al., 1996).

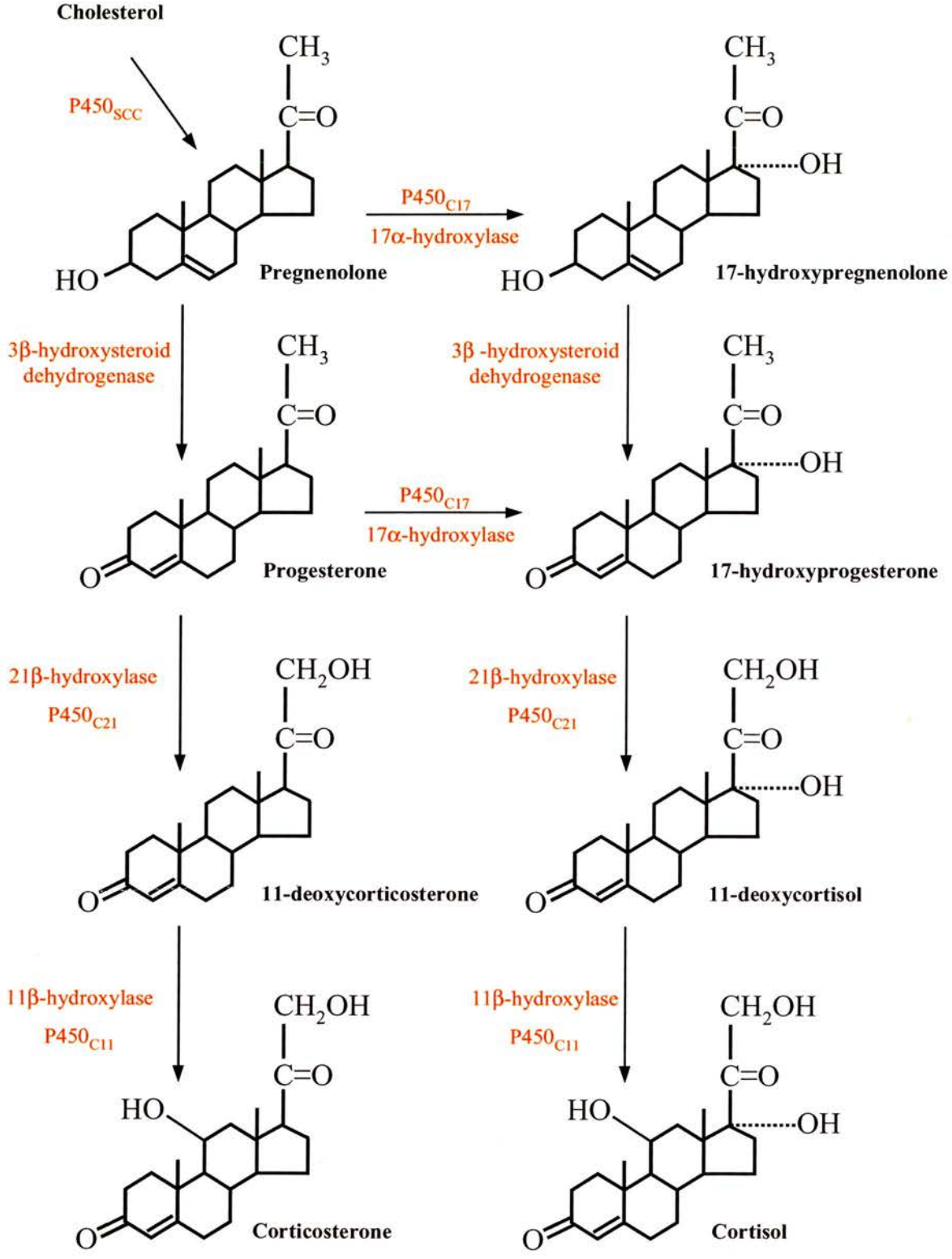
Obviously, the involvement of glucocorticoids in such a large and diverse array of physiological processes means that regulation of their actions must be both tight and complex. The following chapter therefore describes how glucocorticoid action is regulated, with particular emphasis on the role of the glucocorticoid receptor.

1.1.1 Glucocorticoid production and secretion

Glucocorticoids are primarily synthesised in the zona fasiculata and zona reticularis of the adrenal cortex and, like all other steroid hormones, are derived from cholesterol. The first stage in the biosynthetic pathway of all steroid hormones involves a rate-limiting step in which the cholesterol side chain is removed by a dedicated cytochrome P-450 enzyme ($P\text{-}450_{SCC}$) to produce pregnenolone (fig. 1.1). Subsequent reactions result in the production of two glucocorticoids; cortisol and corticosterone. In rodents, corticosterone is the major glucocorticoid due to their lack of adrenal $P\text{-}450_{C17}$ enzyme, while cortisol predominates in humans and other mammals. Glucocorticoids are not stored by the adrenals, but are released immediately into the circulation (Orth et al., 1992).

Glucocorticoid secretion is regulated by hormonal interactions between the hypothalamus, pituitary and adrenals (the 'HPA axis'), and also by neural and other stimuli (fig 1.2). Specifically, neural stimuli cause the parvocellular paraventricular neurons of the hypothalamus to release corticotrophin-releasing hormone (CRH), arginine vasopressin (AVP) and other peptides into the hypophyseal portal circulation. These peptides are then delivered to the anterior pituitary, where they stimulate the production of pro-opiomelanocortin (POMC), the precursor for adrenocorticotrophic hormone (ACTH), which is subsequently secreted into the systemic blood. ACTH acts by binding to specific cell-surface receptors of the adrenal cortex, where most, if not all, of its effects are mediated through cyclic adenosine monophosphate (cAMP). ACTH exerts an acute effect (occurring within minutes) on glucocorticoid biosynthesis by increasing the rate at which cholesterol is converted to pregnenalone by $P\text{-}450_{SCC}$. The chronic effects of ACTH occur over hours and days and involve increased synthesis of most of the enzymes of the steroidogenic pathway, as well as more general actions on adrenocortical protein and RNA synthesis and cell growth (Orth et al., 1992).

Figure 1.1 *Steroid biosynthetic pathways of cortisol and corticosterone in the adrenal cortex.*
Diagram showing the pathways for cortisol and corticosterone production and the structures of these steroids and their biosynthetic precursors. Biosynthetic enzymes are shown in red. SCC, side chain cleavage. Adapted from Orth et al., 1992.

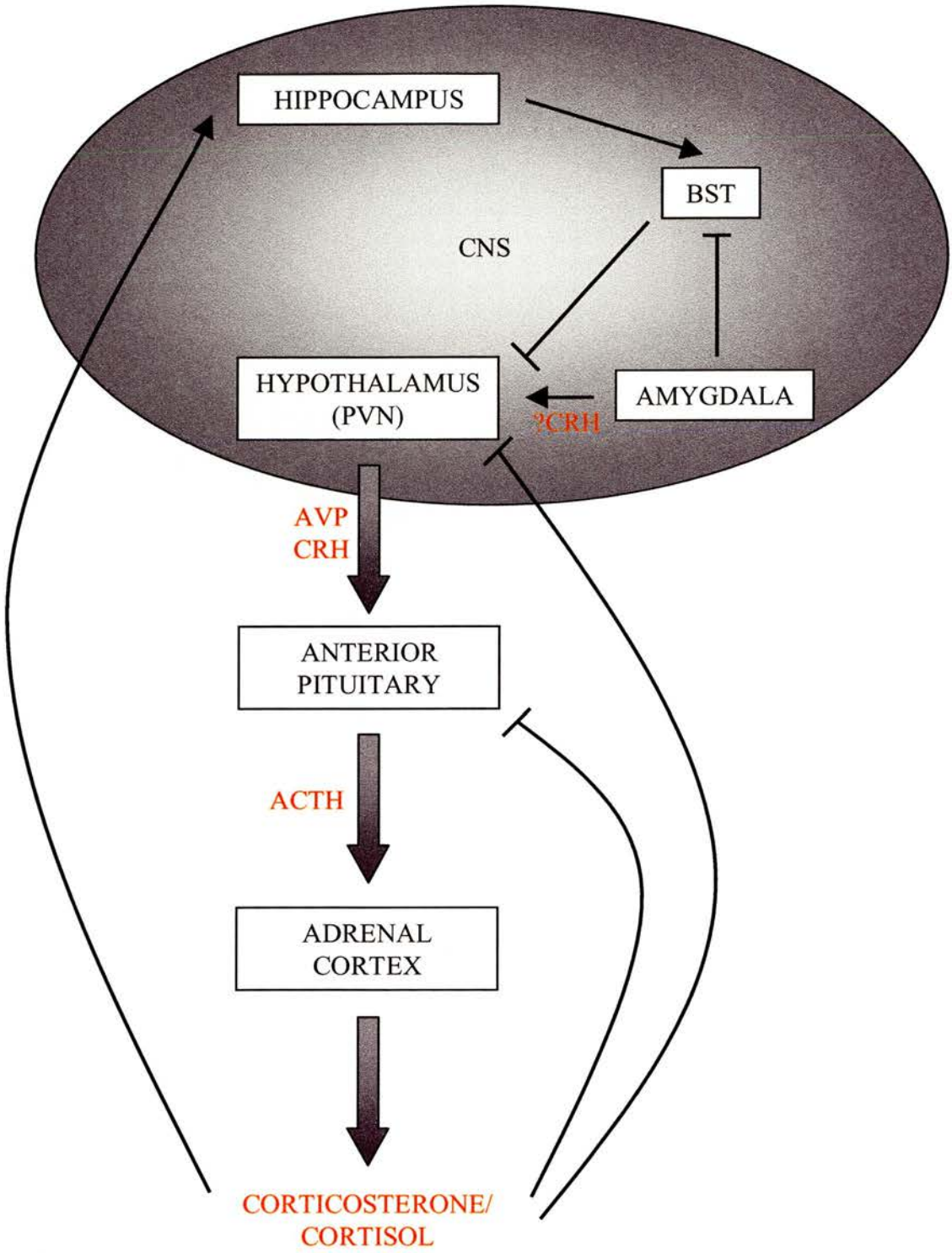

Cholesterol
P450SCC
Pregnenolone
P450C17
17α-hydroxylase
17-hydroxypregnenolone
3β-hydroxysteroid dehydrogenase
Progesterone
3β -hydroxysteroid dehydrogenase
17-hydroxyprogesterone
21β-hydroxylase P450C21
11-deoxycorticosterone
11-deoxycortisol
11β-hydroxylase P450C11
Corticosterone
Cortisol

1.1.2 Regulation of glucocorticoid secretion

An endogenous pacemaker, most probably located in the suprachiasmatic nucleus of the hypothalamus, generates a circadian rhythm in a variety of physiological processes, including HPA axis activity (Orth et al., 1992). As such, levels of ACTH and glucocorticoid secretion reach a peak just prior to the daily period of activity (morning in humans and evening in rats) and decline to their nadir an hour or two after sleep begins. Most importantly, acute physical or psychological stress activates the HPA axis, increasing both ACTH and glucocorticoid secretion (Orth et al., 1992). However, glucocorticoid secretion is autoregulated via a negative feedback loop that occurs at both the pituitary and hypothalamic level. In the pituitary, glucocorticoids inhibit POMC gene transcription and hence ACTH production. In the hypothalamic paraventricular nuclei, glucocorticoids decrease CRH and AVP mRNA and protein levels, as well as blocking the stimulatory effect of CRH on POMC expression and acute ACTH release (Orth et al., 1992).

The hippocampus has also been implicated in the regulation of HPA axis activity. Excitatory neurons project from the hippocampus to the lateral bed nucleus of the stria terminalis (BST), which, in turn, exerts an inhibitory effect on the hypothalamus (Raber, 1998). However, maintenance of basal HPA activity appears to be mediated by MR, rather than GR, although under conditions of elevated glucocorticoid secretion (e.g. after stress or during the circadian peak in glucocorticoid secretion), GR become progressively occupied (De Kloet et al., 1998). It appears that GR and MR have opposing effects on neuronal excitability and hippocampal 'outflow' and that hippocampal GR activation disinhibits tonic maintenance of HPA axis activity by MR (De Kloet et al., 1998). In contrast, the amygdala appears to exert a stimulatory effect on HPA axis activity, principally through inhibition of outflow from the BST, but also through the projection of excitatory neurons directly onto the hypothalamus (Raber, 1998). The involvement of the amygdala in anxiety and fear-related memory are intrinsically relevant to the stress response; after stress, the amygdala increases HPA axis activity and fear memory is strengthened (Raber, 1998)

Figure 1.2 *Regulation of glucocorticoid secretion by the hypothalamic-pituitary-adrenal axis.*
Diagram showing the excitatory (represented by arrows) and inhibitory (represented by blunt arrows) interactions involved in regulation of glucocorticoid secretion by the hypothalamic-pituitary-adrenal axis. The central nervous system (CNS) is shown in grey. Physiological structures are represented by boxes. Secreted peptides and steroid hormones are shown in red. Adapted from Orth et al., 1992.


HIPPOCAMPUS
BST
CNS
HYPOTHALAMUS
(PVN)
AMYGDALA
?CRH
AVP
CRH
ANTERIOR
PITUITARY
ACTH
ADRENAL
CORTEX
CORTICOSTERONE/
CORTISOL

1.1.3 Modulation of glucocorticoid actions

There are several mechanisms by which the actions of glucocorticoids are modulated. Firstly, the amount of glucocorticoids secreted by the adrenal gland can vary according to HPA axis activity (section 1.1.2). Secondly, availability of circulating glucocorticoids is modulated by the corticosteroid binding globulin (CBG) protein (Dunn et al., 1981). *In vivo*, approximately 90% of circulating glucocorticoids are bound to CBG, with the majority of the remainder bound to albumin (Dunn et al., 1981). While it has been assumed that CBG acts as a 'sink', there is also evidence indicating that glucocorticoids only enter cells upon dissociation from CBG (Pardridge, 1987), suggesting that it may be part of a system designed to deliver glucocorticoids to target cells. For instance, high activity receptors for CBG have been found on target cell membranes and cleavage of CBG by a serine protease has been shown to result in release of bound glucocorticoid (Singer et al., 1988; Hammond, 1988). After reaching the plasma membrane of target cells, lipid-soluble glucocorticoids enter cells principally by diffusion, although it is possible that some active transport may occur (Orth et al., 1992).

Thirdly, the availability of glucocorticoids is modulated by their metabolism and excretion. In particular, the 11β-hydroxysteroid dehydrogenase 2 (11βHSD2) enzyme converts active cortisol (corticosterone in rats) to inactive cortisone (11-dehydrocorticosterone in rats), while the 11βHSD1 enzyme catalyses the reverse reaction to generate active glucocorticoids (reviewed in Seckl et al, 2002). In aldosterone target tissues such as the kidney, MR is co-localised with 11βHSD2, with evidence showing that the two molecules can physically interact (Odermatt et al, 2001). In this way, MR, as a high affinity glucocorticoid receptor, is protected from exposure to high glucocorticoid levels, thus allowing it to bind aldosterone (Stewart, et al., 1995). In addition, during development, 11βHSD2 is widely expressed in the both the placenta and foetus during early to mid gestation where it protects the developing foetus from potentially harmful levels of maternal glucocorticoids (Stewart et al., 1995; Seckl et al, 2002). In contrast, 11βHSD1 reactivates inactive glucocorticoids in classical glucocorticoid target tissues such as

liver, brain and adipose fat (Kotelevtsev et al., 1997; Seckl et al, 2002). Excretion of glucocorticoids in the urine and faeces occurs following a variety of reduction, oxidation and hydroxylation reactions (Orth et al., 1992).

1.1.4 Physiological effects of glucocorticoids

1.1.4.1 The stress response

Glucocorticoids affect a variety of physiological processes, but perhaps their most important role is exerted during the stress response. Acute physical or psychological stress increases HPA activity and consequently glucocorticoid secretion (Orth et al., 1992). In the short term, the effects of glucocorticoids promote survival in the face of a stressor by enhancing energy availability and inhibiting non-essential processes such as inflammation, growth and reproduction (McEwen, 2000). However, the negative feedback actions of glucocorticoids are vital in restoring homeostasis following a stressful event (Dallman et al., 1995). This autoregulatory mechanism prevents the pathologies associated with prolonged exposure to elevated glucocorticoid levels, which can include myopathy, osteoporosis, hypertension and diabetes (characteristics of Cushing's syndrome) (McEwen, 2000). In this homeostatic role, glucocorticoids also act to protect the body from over-reactivity of the immune system during illness (section 1.1.4.3).

1.1.4.2 Intermediary metabolism

Glucocorticoids derive their name from their role in glucose metabolism, but they can be seen to regulate virtually all metabolic processes. Following food intake, they promote energy storage in the form of glycogen by activating glycogen synthase and inactivating the glycogen-mobilising enzyme glycogen phosphorylase (Orth et al., 1992). Conversely, glucocorticoids can also increase energy availability during fasting by directly activating key hepatic gluconeogenic enzymes, such as glucose-6-phosphatase (Garland et al., 1986) and phosphoenolpyruvate carboxykinase (PEPCK) (Hanson & Reshef, 1997). They also act to inhibit glucose uptake and

utilisation in peripheral tissues, in part by direct inhibition of glucose transport into these cells (Orth et al., 1992). Finally, glucocorticoids are intrinsically involved in lipid metabolism, where they activate lipolysis in adipose tissue, enhancing free fatty acid release (Orth et al., 1992; Brindley, 1995).

1.1.4.3 Immune system

Glucocorticoids are known to exert gross effects on thymus size in both developing and adult animals (Akana et al., 1985). An experiment carried out in 1924 first showed that adrenalectomy caused significant thymus growth (Jaffe, 1924). More recent studies in which adrenalectomised rats were given replacement corticosterone at various concentrations showed that the range of plasma glucocorticoids compatible with normal thymus mass is very narrow, indicating that thymocytes are extremely sensitive to the apoptotic effects of glucocorticoids (Akana et al., 1985). Further work has shown that, at pharmocological levels, glucocorticoids induce apoptosis in immature thymocytes, but not in mature T cells (Miller et al., 1998). This apoptotic effect is important in the selection of T cells bearing TCRs that recognise self antigens with low-to-moderate avidity, rather than those with near-nil avidity (which would be immunologically useless) or high avidity (which would pose autoimmune problems). While the mechanism mediating this effect is outside the scope of this thesis, it has been the subject of several reviews (see Ashwell et al., 2000 and references therein). However, the effects of glucocorticoids on T cell development and selection in the thymus has been a matter considerable controversy since it was reported that transgenic mice in which GR is completely abolished are born with a normal thymus and normal thymocyte populations (Godfrey et al., 2000; Godfrey et al., 2001; Purton et al., 2000; Jondal et al., 2001; Pazirandeh et al., 2002).

The clinical significance of glucocorticoids lies in their role as powerful immunosuppressors and, as such, they are among the most widely prescribed drugs in the world. One of the principal effects of glucocorticoids is on the traffic of immune cells to and from peripheral circulation. In particular, glucocorticoids are associated with depletion of peripheral lymphocytes, with a greater reduction

observed in T cells than B cells. Peripheral levels of both monocytes and neutrophils are also reduced, leading to suppression of local inflammatory responses (reviewed in McEwen et al., 1997). Most importantly, glucocorticoids inhibit the actions of both nuclear factor kappa B (NF-κB) and AP-1, transcription factors that play key pro-immflammatory roles in both B- and T-cells (Elenkov & Chrousos, 1999; Barnes & Adcock, 1998). NF-κB induces the expression of a wide range of cytokines and chemokines, including IL-1, IL-2, IL-6, TNFα, β- and γ-interferons, as well as TCRs α and β (reviewed in McKay & Cidlowski, 1999) while AP1 is known to induce genes involved in inflammatory diseases such as asthma (Barnes & Adcock, 1998). The mechanisms behind the inhibitory actions of glucocorticoids on both NF-κB and AP-1 remain, to a large extent, unresolved. However, the transrepression mechanism is mediated by GR, and has been postulated to involve direct interactions with NF-κB and AP-1, effects on I-κB or through the recruitment of co-activators (discussed in further detail in section 1.2.1.5).

1.1.4.4. Developmental effects

While glucocorticoids are known to promote the development of many tissues, one of their most critical functions involves lung maturation, in which they induce morphological changes and surfactant production in type II pneumocytes (Orth et al., 1992). In fact, transgenic mice in which GR is completely abolished die within a few minutes of birth due to atalectasis of the lungs (Tronche et al., 1998) (see section 1.2.2.2). The mechanisms by which glucocorticoids induce lung maturation remain obscure. Initial evidence suggested that glucocorticoid-induced effects on lipid metabolism caused the release of phosphatidyl and fatty acids which, in turn, activated a key enzyme in surfactant synthesis, cholinephosphate cytidyltransferase (Mallampalli et al., 1994). However, more recent studies in GR knock out mice have indicated that deficient surfactant production is not the cause of their lethal phenotype (Kellendonk et al., 1999).

In the nervous system glucocorticoids regulate the differentiation of neural crest epithelial cells into chromaffin cells (Anderson et al., 1993). Under the influence of

glucocorticoids, neural crest precursor cells invade the embryonic adrenal gland and cease to express neuron-specific gene products such as neurofilaments and instead acquire the characteristics of adrenomedullary chromaffin cells (Anderson, 1993). The most notable of these characteristics is the ability of differentiated chromaffin cells to synthesise phenylethanolamine-N-methyl transferase (PNMT), the enzyme that converts noradrenaline to adrenaline (Anderson et al., 1993). This model was supported by initial analysis of GR-deficient mice in which GR appeared to be necessary for the formation of adrenal chromaffin cells (Cole et al., 1995). However, subsequent investigations showed that mice carrying targeted GR mutations possessed normal chromaffin cell numbers, but were unable to produce PNMT (Finotto et al., 1999).

1.1.4.5 Neuropsychiatric and behavioural effects

Most brain regions, including the cerebellum, amygdala, cortex, thalamus and hypothalamus, express GR and are therefore sensitive to glucocorticoids (Herman et al., 1989). However, the highest levels of GR expression are seen in the hippocampus, where the lack of 11βHSD2 means that hippocampal MRs also effectively function as glucocorticoid receptors (Arriza et al., 1988; De Kloet et al., 1998). The hippocampus plays key roles in memory, mood and behaviour, all of which are affected by local glucocorticoid exposure (Lupien & McEwen, 1997). For instance, while low levels of glucocorticoids (sufficient to activate MR but not GR) have been shown to enhance memory (De Kloet et al., 1998), stress-induced elevation of glucocorticoids and GR agonists appears to attenuate memory (Seckl & Olsson, 1995). In addition, patients with major depressive disorders tend to hypersecrete cortisol and normalisation of HPA axis activity through anti-depressant treatment has been shown to significantly improve outcome (Seckl & Olsson, 1995). The involvement of the amygdala in behavioural responses to fear and anxiety are mediated, at least in part, by CRH, which is secreted by both the amygdala itself and the hypothalamus (Raber, 1998). Glucocorticoids increase CRH expression in the amygdala thereby enhancing anxiety (Hsu et al., 1998), while adrenalectomy decreases CRH expression (Palkovits et al., 1998).

1.2 The Glucocorticoid Receptor

The physiological effects of glucocorticoids and indeed, the negative feedback loop that regulates their secretion are all mediated by GR. As such, both the mechanism and regulation of GR action are crucial in determining cellular responses to circulating glucocorticoids.

In virtually all species examined to date, only a single GR gene has been identified. In the human, the GR gene is located on chromosome 5 (Gehring et al., 1985) and consists of nine exons (fig 1.3) spread over ~124kb of DNA (Encio & Detera-Wadleigh, 1991). All ligand activated transcription factors, including GR, possess a modular structure consisting of a ligand-binding domain (LBD), a DNA-binding domain (DBD) and one or more activation domains (reviewed in Beato et al., 1995). These functional domains are transposed onto the genomic structure of the GR locus. Exon 1 and the first part of exon 2 contain the 5'-leader sequence, exon 2 encodes the amino-terminal portion, exons 3 and 4 separately encode the two putative zinc fingers that form the DBD, and exons 5-9 combine to form the LBD (Encio & Detera-Wadleigh, 1991). Studies in mouse (Strahle et al., 1992) and rat (Gearing et al., 1993), as well as over a dozen species including several non-human primates, guinea pig, African frog, rainbow trout, flounder, chicken, sheep, pig and others (see http://nrr.georgetown.edu/GRR/GRR.html and references therein), have shown that this structure is highly conserved across species.

1.2.1 Mechanism of GR action

1.2.1.1 Ligand binding and dissociation from heat shock proteins

In the absence of ligand, GR forms a large heteromeric complex with several other proteins, from which it dissociates upon ligand binding (reviewed in Pratt & Toft, 1997). One of the most important components of this complex is hsp90, which associates with the LBD and keeps the receptor in a conformation that is able to bind steroid but is transcriptionally inactive (Cadepond et al., 1991). Studies have also

identified factors including p23, p60, hsp40, hsp70, as well as immunophilins such as hsp56, in the stable GR-hsp90 heterocomplex (reviewed in Schaaf & Cidlowski, 2003).

The crystal structure of the human GR ligand binding domain (LBD) bound to dexamethasone and a coactivator motif derived from the transcriptional intermediary factor 2 has been elucidated (Bledsoe et al., 2002). Despite structural similarity to other steroid receptors, the GR LBD adopts a surprising dimer configuration involving formation of an intermolecular beta sheet (Bledsoe et al., 2002). The structure also reveals an additional charge clamp that determines the binding selectivity of a coactivator and a distinct ligand binding pocket that explains the selectivity of GR for endogenous steroid hormones (Bledsoe et al., 2002). The functional importance of the LBD structure has been demonstrated by studies in which mutation of single amino acid residues induce both decreases and increases in binding affinity, as well as changing the specificity for different ligands (Hurley et al., 1991) (see Schaaf & Cidlowski, 2003 and references therein). Similarly, clinical studies have shown that patients carrying mutations in the GR LBD exhibit marked symptoms of glucocorticoid resistance (table 1.1)

	Polymorphism	Manifestation	Reference
A	D641V (LBD)	Familial glucocorticoid resistance	(Karl & Chrousos, 1993)
B	4bp deletion at exon/intron 6 splice site (LBD)	Glucocorticoid resistance	(Karl et al., 1993)
C	L753F (LBD)	Glucocorticoid resistance; human leukaemic CCRF-CEM cells	(Powers et al., 1993) (Palmer & Harmon, 1991)
F	D559I, V729I (LBD)	Familial glucocorticoid resistance	(Hurley et al., 1991)

Table 1.1 *Summary of GR polymorphisms in the LBD*
Single amino acid nomenclature; D, aspartic acid; V, valine; L, leucine; F, phenylalanine; I, isoleucine. Adapted from (Yudt & Cidlowski, 2002).

The relationship between the GR LBD structure and glucocorticoid-resistance is further exemplified by the guinea-pig. The guinea-pig is a well-documented cortico-resistant species, a characteristic that is caused by differences within the amino-terminal portion of the GR LBD (Keightley et al., 1998). However, it should be

noted that the GR LBD has not been implicated in the glucocorticoid-resistance exhibited by several New World primates, including the squirrel monkey and the marmoset (Brandon et al., 1995). In these species, resistance has been linked to a cytosolic factor that somehow reduces sensitivity to circulating glucocorticoids (Brandon et al., 1995).

1.2.1.2 Phosphorylation and nuclear translocation

GR is phosphorylated in the absence of hormone and becomes hyperphosphorylated after binding of an agonist, but not an antagonist (Orti et al., 1989) (see section 1.2.2.1.3). The phosphorylated GR subsequently translocates to the nucleus. However, receptor phosphorylation is not necessary for nuclear translocation since results show that a mouse GR lacking all phosphorylation sites still undergoes nuclear translocation upon ligand activation (Webster et al., 1997). Two domains of GR have been implicated in nuclear translocation; the first (NL1) is located in the C-terminal portion of the DBD and is 100% conserved between human and rat; the exact location of the second (NL2) is unknown, although it has been mapped to the LBD (Picard & Yamamoto, 1987).

1.2.1.3 Dimerisation and DNA binding

The effects of GR on gene transcription are mediated in most cases through binding to specific DNA sequences that form glucocorticoid response elements (GREs) (Beato et al., 1989). This interaction was originally thought to be dependent upon the GR DNA binding domain (DBD), which consists of two protein loops co-ordinated by a zinc ion, resulting in two zinc fingers that are each followed by an amphipathic helix (Luisi et al., 1991). Under the traditional model, a GRE, comprised of a partially/completely palindromic pair of DNA 'half-sites', binds GR as an obligate homodimer (Luisi et al., 1991). This appears to occur through initial binding of a GR monomer to the higher-affinity half-site, followed by co-operative binding of a second monomer, which is stabilised by protein-protein interactions through a 'dimerisation interface' within the second zinc finger of the DBD (Dahlman-Wright

et al., 1991). Further stability is achieved when GREs are multimerised, which probably provides the basis for the transcriptional synergy observed in GR (Strahle et al., 1988; Schmid et al., 1989).

However, very recent work has shown that the model described above does not apply to a subset of GREs, at which dimerisation appears to be unnecessary (Adams et al., 2003). The implications of DNA dimerisation deficiency are discussed in more detail in section 1.2.1.6.

1.2.1.4 Transactivation

Transactivation of target genes has been shown to be dependent upon two domains of GR; τ1 and τ2 (Hollenberg & Evans, 1988). The first is located in the N-terminal region of the receptor and its function is hormone-independent, while the second maps to the LBD and requires hormone binding for optimal activity (Giguere et al., 1986). The exact mechanism of how binding of GR to a GRE leads to transcription initiation is unknown, although GR itself is capable of recruiting and stabilising the transcriptional pre-initiation complex (Freedman, 1999). In addition, GR has been shown to make contacts with numerous components of the transcriptional machinery *in vitro* (Robyr et al., 2000). However, transactivation by GR is also known to be dependent upon co-activators that facilitate either the recruitment of basal transcription machinery or the remodelling of chromatin.

Investigation of the mouse mammary tumour virus long terminal repeat (MMTV LTR), which contains several GREs, has shown that transcriptional activation by GR requires DNA binding, disruption of local chromatin structure and assembly of the initiation complex at a TATA box (Hebbar & Archer, 2003). The GR homodimer recognises adjacent major grooves on only one face of the DNA and, as such, GR binding to nucleosomal DNA is less affected by steric hindrance that other transcription factors (Deroo & Archer, 2001). GR has been shown to associate with BRG-1, a key component of the BRG-1/BAF complex (homologous to the yeast SWI/SNF complex) that acts as an ATP-dependent chromatin-remodelling machine

(Fryer & Archer, 1998). BRG-1 removes histone H1 from the chromatin, thus allowing general transcription factors, including nuclear factor (NF)-1 and TBP, to access their binding sites (Fryer & Archer, 1998).

Interestingly, the interaction between GR and the MMTV LTR locus is consistent with a dynamic model ('hit and run'), in which GR first binds to the chromatin after ligand activation, recruits remodelling activity and is then lost from the template (Hager et al., 2000) (McNally et al., 2000). However, studies in which GR induces the formation of nuclease-sensitive chromatin at an enhancer of the rat tyrosine aminotransferase (TAT) gene show that ongoing enhancer activity requires continued interaction between GR and its agonist (Reik et al., 1991). However, while chromatin remodelling appears to be reversible after hormone withdrawal, more recent work has shown that GR-induced DNA demethylation at the enhancer provides a 'memory' of a glucocorticoid regulatory event during development (Thomassin et al., 2001).

1.2.1.5 Transrepression

In addition to its role as an activator of transcription, GR can also repress the expression of specific target genes by at least three putative mechanisms. Firstly, it can bind to DNA sequences called negative GREs (nGREs), which are present in the promoters of genes such as osteocalcin and prolactin (see Schaaf & Cidlowski, 2003 and references therein). While the sequences of nGREs are too heterogeneous to determine a consensus, binding of GR presumably replaces and/or prevents binding of a transactivation transcription factor (Drouin et al., 1989). Of particular note is the nGRE in the POMC promoter, on which a complex of three GR molecules induces transrepression (Drouin et al., 1989). Specifically, a GR homodimer binds to one side of the element while a GR monomer binds to the other (Drouin et al., 1989).

Secondly, GR can bind to DNA elements consisting of a non-overlapping GRE and a binding site for a different transcription factor (known as composite GREs). This is the case for the proliferin promoter, where the composite GRE includes an AP1

binding site (Malkovski et al., 1999). Interestingly, GR can enhance or repress AP1-induced transactivation depending upon cell type and composition of the AP1 dimer (Pearce & Yamamoto, 1993).

The final mechanism of transrepression occurs without the involvement of DNA binding by GR. For example, AP1-induced activation of the collagenase promoter is inhibited by ligand-activated GR, probably through a protein-protein interaction. The AP1 binding site is recognised by Jun homodimers and Jun/Fos heterodimers and GR can interact directly, via its DBD, to both (although Fos appears to be the preferred target) (Yang-Yen et al., 1990). In this way, AP1 and GR can be seen to reciprocally antagonise the transactivity of each other (Yang-Yen et al., 1990). In addition, activated GR has been shown to repress activity of NK-κB, specifically through direct interaction of its DBD with the p65/RelA subunit (McKay & Cidlowski, 1999). These examples of reciprocal antagonism may well be related to the opposing effects of AP1/NF-κB and GR upon inflammatory responses (McKay & Cidlowski, 1999). Furthermore, an additional mechanism has been proposed based on the observation that in some cells, glucocorticoids are known to induce expression of members of the I-κB family, which sequester NF-κB in the cytoplasm and thus prevent activation of the NF-κB target genes (Auphan et al., 1995).

1.2.1.6 Protein-protein versus protein-DNA interactions by GR

Elucidation of the DNA binding versus the protein-protein interaction roles of GR has been investigated using transgenic mice carrying a point mutation in the DBD. Specifically, $GR^{dim/dim}$ mice were generated in which the D-loop of the second zinc finger (which forms the dimerisation interface) carried a point mutation, thus impairing dimerisation and thus binding to DNA targets (Reichardt et al., 1998). The most striking phenotypic characteristic of $GR^{dim/dim}$ mice was their survival past birth, causing the authors of the report to declare that 'DNA binding is not essential for survival' (Reichardt et al., 1998). These mice showed impaired GR-dependent activation of transcription, as demonstrated by the lack of responsiveness of TAT (which carries a GRE) to glucocorticoid exposure (Reichardt et al., 1998). In

contrast, the glucocorticoid-dependent protein-protein interactions known to repress AP-1 activity were unaffected (Reichardt et al., 1998). Furthermore, $GR^{dim/dim}$ mice revealed that feedback control of the HPA axis is dependent upon both the DNA-binding and protein-protein actions of GR (Reichardt et al., 1998). As such, CRF expression was not-upregulated in transgenic mice, indicating that repression of CRF synthesis by GR does not require DNA binding, but POMC (the gene encoding ACTH) expression was strongly increased and is therefore dependent upon GR DNA binding activity (Reichardt et al., 1998).

However, as mentioned previously (section 1.2.1.3), very recent work has indicated that the GR^{dim} mutation only abolishes GR binding at a subset, and not all, of GREs (Adams et al., 2003). Results showed that GR binding and transactivation at 'simple', single GREs, including those found in the promoters of the TAT and MMTV LTR genes, are indeed dependent upon an intact dimer interface. However, promoters containing more complex arrangements of GRE consensus half- and partial- sites, including that of PNMT, can be both bound and activated by GR^{dim} (Adams et al., 2003). Under this model, the GR-dependent functions that are intact in $GR^{dim/dim}$ mice depend upon the formation of 'concerted GR multimers' on target promoters, which interact at complex arrays of GREs and are supported by various protein-DNA and protein-protein interactions (Adams et al., 2003). It is therefore conceivable that DNA binding by GR might indeed be essential for survival. Further transgenic models, in which GR is unable bind to DNA at all, must be developed to further investigate this intriguing problem.

1.2.2. Regulation of GR action

1.2.2.1 Isoforms of GR

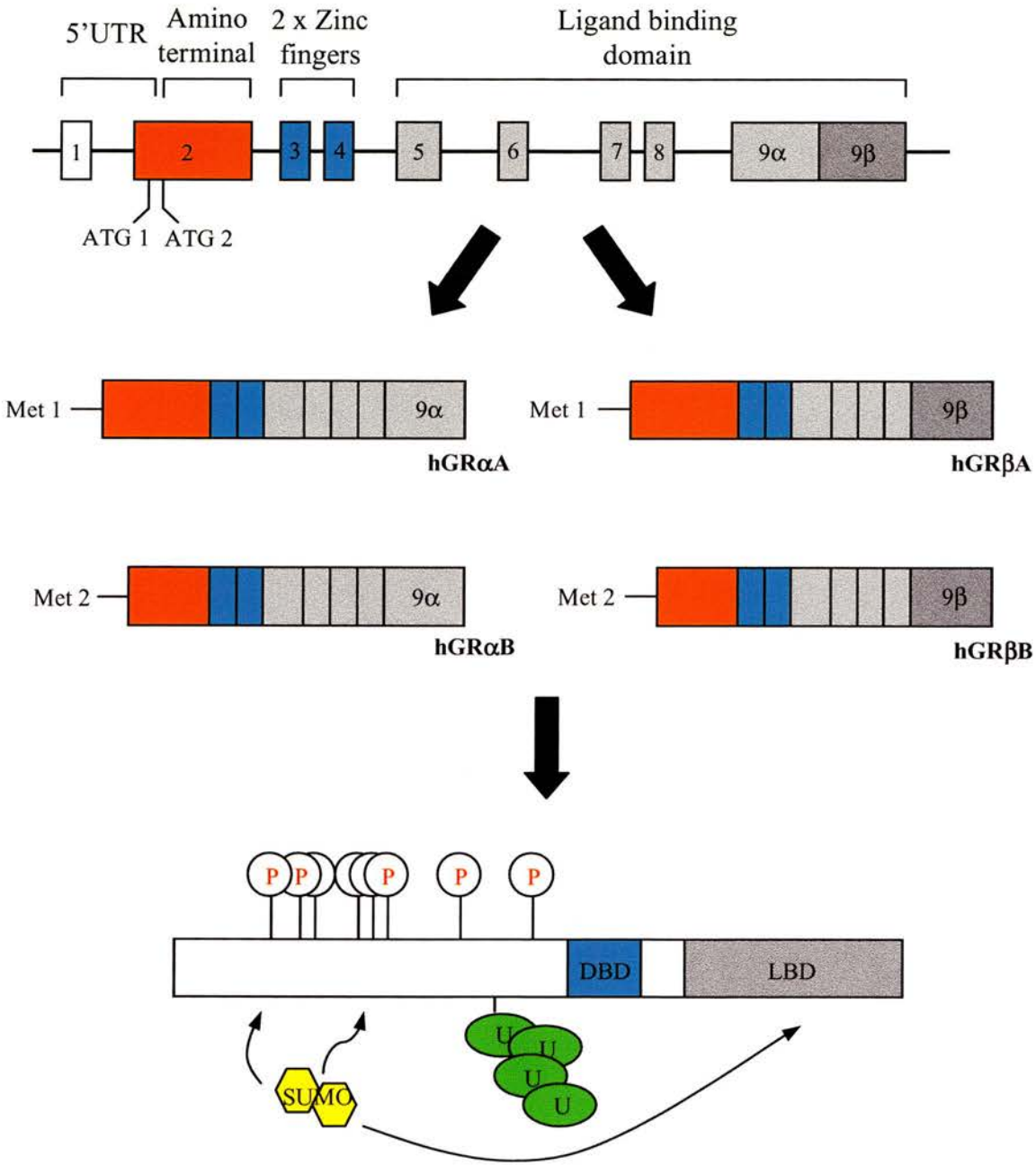
It is evident that GR exerts a complex range of actions on a variety of target genes. This complexity arises, in part, through diversity of GR isoforms. While until very recently only a single GR gene had been identified in all species examined, studies in carried out in a cichlid fish, *Haplochromis burtoni*, have identified two GR isoforms,

termed HbGR1 and HbGR2, which are encoded by separate genes (Greenwood et al., 2003). Phylogenetic analysis indicates that the two GR paralogues are unique to fish and are the result of a gene or genome duplication event (Greenwood et al., 2003). The difference between the isoforms occurs at the 3'-UTR, which is shorter in HbGR1 than in HbGR2. Interestingly, the transactivation properties of the two isoforms were shown to differ when tested on a promoter containing three classical GREs in tandem, with HbGR1 eliciting a higher level of reporter gene expression than HbGR2 (Greenwood et al., 2003). However, in all tissues tested, including brain, heart, gill, kidney, liver and spleen, levels of HbGR2 mRNA exceed those of HbGR1 (Greenwood et al., 2003). It is possible that the shortened 3'-UTR of HbGR1 mRNA renders it less stable, which may account for its decreased presence in all tissues examined. As such, it remains to be seen if the increased transactivation properties of HbGR1 are functionally significant.

1.2.2.1.1 GRα and GRβ

When a human GR cDNA was first cloned in 1985, two gene splicing products were identified and termed hGRα and hGRβ (reviewed in Schaaf & Cidlowski, 2003). Immediately, hGRα was recognised as the classical GR, composed of a single polypeptide chain of 777 amino acids and the primary mediator of glucocorticoid action (Hollenberg et al., 1985). hGRβ is generated through alternative splicing of the ninth and final exon (fig 1.3), resulting in a protein sequence divergent only at the carboxy terminal (Oakley et al., 1996). As such, while hGRα and hGRβ are identical through to amino acid 727, the 15 carboxy-terminal amino acids of hGRβ are unique and replace the carboxy-terminal 50 amino acids in hGRα. In all systems tested to date, hGRβ is transcriptionally inactive and is unable to bind either agonists or antagonists (Oakley et al., 1996; Yudt & Cidlowski, 2002). Furthermore, unlike hGRα, the hGRβ isoform is located in the nucleus in the absence of ligand (Oakley et al., 1996; Yudt & Cidlowski, 2002). These features led hGRβ to originally be dismissed as physiologically irrelevant. However, more recent studies have indicated that the isoform may exert a dominant-negative effect on hGRα-mediated

Figure 1.3 *Isoforms of human GR.*
Diagram (not to scale) showing the exonic structure of human GR and the generation of various isoforms. Approximate phosphorylation sites are represented by 'P'; 'SUMO' and 'U' represent the actions of sumoylation and ubiquitination respectively. Met, methionine; DBD, DNA binding domain; LBD, ligand binding domain. Adapted from Yudt & Cidlowski, 2003.


5'UTR
Amino terminal
2 x Zinc fingers
Ligand binding domain
1
2
3
4
5
6
7
8
9α
9β
ATG 1
ATG 2
Met 1
9α
hGRαA
Met 1
9β
hGRβA
Met 2
9α
hGRαB
Met 2
9β
hGRβB
P
P
P
P
P
DBD
LBD
U
U
U
U
SUMO

transactivation (Bamberger et al., 1995). Studies into the mechanism of this dominant-negative activity suggest that it is the formation of hGRα/hGRβ heterodimers, incapable of binding co-activators in the same way as ligand activated hGRα homodimers, that causes the observed attenuated hGRα response (Oakley et al., 1999).

Data suggesting a pathophysiological role for hGRβ includes the association of several inflammatory conditions with elevated expression of hGRβ in particular cells (Leung et al., 1997; Christodoulopoulos et al., 2000; Honda et al., 2000). In addition, a polymorphism in the hGRβ 3'UTR, which stabilises the mRNA and increases hGRβ protein levels, has been found in patients suffering from rheumatoid arthritis (DeRijk et al., 2002). However, it should be noted that the physiological relevance of hGRβ is still a matter of some controversy. Firstly, transient transfection studies have suggested that levels of hGRβ must be in excess by at least 5-fold relative to hGRα in order to achieve a significant dominant negative effect, yet all tissues examined express much higher levels of endogenous hGRα (Oakley et al., 1997). Secondly, several groups have been unable to replicate the dominant negative activity of hGRβ (Hecht et al., 1997; de Lange et al., 1999). Finally, although the presence of hGRβ has recently been demonstrated in rat (Korn et al., 1998) no mouse homologue has been identified (Otto et al., 1997).

1.2.2.1.2 GR-A and GR-B

Alternative translation initiation also generates two distinct GR translational isoforms, termed GR-A and GR-B (Yudt & Cidlowski, 2001) (fig. 1.3). The major protein product, with an apparent molecular mass of 94kDa, represents translation from the first initiator AUG codon and is designated GR-A. However, this start codon lies within a weak Kozak sequence, resulting in leaky ribosomal scanning and translation from a downstream start codon (met 27 in human or met 28 in rodents), which generates a 91kDa protein termed GR-B (Yudt & Cidlowski, 2001). Evidence suggests that the shorter GR-B species is nearly twice as efficient in GRE-mediated

transactivation as the longer GR-A, although the biological significance of this finding is still unclear (Yudt & Cidlowski, 2001).

1.2.2.1.3 Post-translational modifications of GR

GR is a substrate for several kinases and phosphatases and is poly-phosphorylated on serine and threonine residues in the amino-terminal region of the protein (Bodwell et al., 1991; fig. 1.3). GR phosphorylation appears to be strongly cell-cycle dependent (Bodwell et al., 1998) and although the precise role of each specific phosphorylation event remains unclear, mutation of multiple phosphorylation sites has been shown to profoundly affect receptor stability, protein half-life and signalling (Webster et al., 1997). There is also data suggesting that mouse GR is a target for both post-translational ubiquitination (which targets the receptor for proteosomal degradation) and sumoylation (which modulates transcriptional regulation) (reviewed in Yudt & Cidlowski, 2002) (fig. 1.3).

1.2.2.1.4 Non-genome-active isoforms of GR

A notable paradox of glucocorticoid biology is the rapid release of glucocorticoid hormone during a stress response (seconds) and the perceived delay (hours) in eliciting a genomic response consistent with the classical action of steroid and other nuclear hormone receptors. In recent years, there has been increased interest in potential non-genomic and/or membrane-associated actions of glucocorticoids and GR. One hypothesis involves a membrane-bound/associated form of GR, termed mGR, which is postulated to be a modified form of the classical GR (Gametchu et al., 1991), although this remains a major point of contention. Interestingly, studies have shown that mGR is more strongly correlated with GC-evoked lymphocytolysis than the classical GR (Sackey et al., 1997).

Other work has proposed that the non-genomic effects of glucocorticoids are transduced by the same biochemical effector pathways responsible for mediating rapid responses to neurotransmitters (Borski, 2000) and implicated second messenger

cascades involving phospholipase C, phosphoinositide turnover, intracellular pH and calcium, protein kinase C, and tyrosine kinases (Wehling, 1997). Considerably more work is required to resolve this paradox, particularly with regard to the proposed mGR mechanism.

1.2.2.2 Regulation of GR levels

Obviously, the most important regulator of cellular sensitivity to glucocorticoids and subsequent GR action is the level of GR itself. This has been demonstrated *in vitro* using rat hepatoma-derived cell lines expressing different levels of GR (Vanderbilt et al., 1987). Results showed that the extent of structural chromatin alteration at a MMTV GRE and the magnitude of several transcriptional responses elicited by the receptor (including those of TAT and α-1-acidic glycoprotein) are roughly proportional to the number of GR molecules per cell (Miesfeld et al., 1986; Vanderbilt et al., 1987).

Furthermore, several groups have developed genetic tools to study GR function *in vivo*. Near-ubiquitous expression of a GR antisense mRNA (under the control of a neurofilament promoter) in mouse tissues caused a 40-60% reduction of GR protein *in vivo* and resulted in increased HPA axis activity (Pepin et al., 1995). In addition, these transgenic animals showed a greatly increased adipose fat deposition (Pepin et al., 1995). However, this approach is limited due to the fact that gene function is only partially inactivated and the precise degree of GR loss at a cellular level is difficult to assess.

Gene targeting approaches have generated two distinct disruptions of the GR gene. The first involved the insertion of a neomycin cassette into the second GR exon and resulted in a hypomorphic allele, GR^{hypo} (Cole et al., 1995). The persistence of mRNA species encoding an amino-terminal truncated protein containing the DBD and LBD meant that this transgenic model was not a complete GR knock out (Cole et al., 2001). The second involved a deletion of the third exon (encoding the first zinc finger of the DBD) and caused complete inactivation of the GR gene, GR^{null}

(Tronche et al., 1998). Both $GR^{hypo/hypo}$ and $GR^{null/null}$ mice reportedly show similar phenotypic changes, although those exhibited in the latter appear to be more severe (Tronche et al., 1998). While both types of mutant mice die shortly after birth due to atalectasis of the lungs, further changes have also been identified. These include the impaired induction of gluconeogenic enzymes, the abolishment of T-cell apoptosis and the reduced proliferation of erythroid progenitor cells (Bauer et al., 1999). Furthermore, while the HPA axis is not fully functional at birth, $GR^{null/null}$ mutant mice showed a significant increase in glucocorticoid (3-fold) and ACTH (15-fold) levels (Tronche et al., 1998). These increases were due to enhanced transcription of the CRF and POMC genes in the hypothalamus and anterior lobes of the pituitary respectively (Cole et al., 1995; Tronche et al., 1998). It appears, therefore, that the HPA axis is already responsive at birth and that GR levels are important in mediating transcriptional repression associated with glucocorticoid negative feedback.

To circumvent the early lethality of $GR^{null/null}$ mice and the associated difficulty in analysing GR function in adult animals, tissue-specific GR mutant mice were generated. Conditional mutations of the GR gene were obtained by employing the Cre/loxP system and have been used to evaluate the role of GR in the nervous system (Tronche et al., 1999; Kellendonk et al., 2002), liver and thymus of adult animals (unpublished data). The targeted absence of GR in the nervous system was not lethal, but the lack of glucocorticoid feedback profoundly altered HPA axis equilibrium (Tronche et al., 1999). The increased circulating glucocorticoid levels in these animals caused them to display symptoms associated with Cushing's disease, i.e. growth retardation, reduced size, osteoporosis and a characteristic 'buffalo hump' fat deposition (Tronche et al., 1999), although further work showed that fat deposits decreased after weaning (Kellendonk et al., 2002). Interestingly, these mice also appear to be less anxious (Tronche et al., 1999; reviewed in Gass, 2001).

While the studies described above investigated the effects of hypomorphic or null GR expression, the effects of increased GR gene dosage have also been studied. These investigations involved mice in which a yeast artificial chromosome was used to generate animals carrying two additional copies of the GR gene. As might be

expected, these mice show altered basal regulation of the HPA axis, resulting in reduced expression of CRH and ACTH and a fourfold reduction in circulating glucocorticoids (Reichardt et al., 2000). These mice showed a weaker response to restraint stress than wild type controls (Reichardt et al., 2000), possibly due to increased sensitivity and thus more rapid attenuation of the HPA axis. In addition, primary thymocytes taken from these transgenic mice showed an enhanced sensitivity to glucocorticoid-induced apoptosis (Reichardt et al., 2000).

1.2.2.2.1 Homologous down-regulation of GR

Levels of GR are widely recognised to undergo down-regulation after exposure to glucocorticoids in most cells and tissues (see Burnstein et al., 1991 and references therein). While this mechanism once again serves to protect the body from over-reactivity of the glucocorticoid response, this reduction in cellular GR levels also leads to insensitivity to subsequent hormone administration and, as such, has important clinical implications. The mechanisms of autoregulation have been studied extensively both *in vitro* and *in vivo*, and, although reports have drawn conflicting conclusions, results consistently indicate that both GR mRNA and protein levels are affected.

Nuclear run-on experiments have demonstrated a dexamethasone-induced reduction in GR transcription rate in H4IIE cells and liver (Dong et al., 1988) as well as in IM-9 (human B cell), AR42J (rat pancreatic acinar) (Rosewicz et al., 1988) and in transfected COS-1 (green monkey kidney) cells (Burnstein et al., 1994). The most obvious explanation for this ligand-induced reduction in transcription would be repression of the activity of the GR promoter. Indeed, a region of the human GR promoter, shown to bind the novel transcription factor GRF-1, has been implicated in down-regulation of a transiently transfected hGR-CAT reporter construct (Govindan et al., 1991; Leclerc et al., 1991a; Leclerc et al., 1991b). Furthermore, GRF-1 mRNA was shown to be up-regulated in rat liver following dexamethasone treatment (Leclerc et al, 1991b), indicating that increased levels of GRF-1 protein bind to the human GR promoter and consequently repress transcription. However, this result is

contradicted by several studies showing that homologous down-regulation of GR mRNA and protein appears to be independent of protein synthesis, since it occurs in the presence of cylcohexamide (Okret et al., 1986; Burnstein et al., 1994).

Several studies also suggest that repression of GR expression may occur independently of the GR promoter. Transfection studies in which GR expression vectors were under the control of either the Rous sarcoma virus (Burnstein et al., 1991) or human metallothionein-IIa (Alksnis et al., 1991) promoters, also showed a decrease in GR mRNA after steroid treatment. Therefore, it was suggested that sequences outside the GR promoter might also be involved in homologous down-regulation. Accordingly, investigations have shown that GR can bind to both a 2.6kb fragment of the 3'-UTR of GR cDNA (Okret et al., 1986) and a region encoding amino acids 550-697 of the ligand binding domain of GR cDNA (Burnstein et al., 1994). However, these results must be considered with the caveat that cDNA does not physiologically exist in cells.

With regard to the effect of glucocorticoids on GR mRNA stability, several groups report no effect in human lymphocytes (Rosewicz et al., 1988) or rat hepatoma cells (Dong et al., 1988), while others describe a greater than 2-fold increase in GR mRNA degradation in transfected COS-1 cells (Burnstein et al., 1994). Finally, there is conflicting evidence on the effect of glucocorticoids on the half-life of the GR protein; reports describe a marked decrease in rat hepatoma derived cell lines, rat liver (Dong et al., 1988) and GH1 cells (McIntyre & Samuels, 1985), while other groups observed no effect in IM-9 human lymphocytes, rat pancreatic acinar AR42J cells (Rosewicz et al., 1988) or NIH3T3 cells (Hoeck et al., 1989).

Previous studies have presented conflicting evidence of the short, medium and long-term effects of glucocorticoids on GR expression levels. Studies in rat hepatoma cells, show a small, initial increase in GR mRNA after 6h of dexamethasone treatment, followed by a 50-95% decrease after 24h, with initial levels restored after 72h (Okret et al., 1986). However, the significance of the observed initial up-regulation in GR mRNA following dexamethasone treatment has been reduced by *in*

vivo experiments in rat liver that did not show any early increase in either GR mRNA or protein levels (Dong et al., 1988). Similarly, another report described a continued reduction in GR mRNA levels in a variety of rat tissues for up to 72 hours, after which there was no further decrease, indicating a 'plateau effect' (Kalinyak et al., 1987).

Autoregulation of GR levels *in vivo* is demonstrated by transgenic mice carrying two additional copies of the GR gene (using a yeast artificial chromosome) which did not show the predicted two-fold elevation of GR expression levels (Reichardt et al., 2000). Instead, the highest levels of over-expression were observed in the brain and the pituitary (60% and 43% respectively), whereas in the spleen, thymus and liver, GR was over-expressed by only 20-24%. Mice carrying only one copy of the GR gene also express GR at levels greater than 50%, with levels of between 70 and 100% observed in most tissues (Cole et al., 2001). This is perhaps the strongest evidence that GR levels are autoregulated and also indicates that, in the long term, autoregulation exerts differential effects between tissues.

Finally, while in most cells and tissues, GR levels have been observed to decrease following glucocorticoid exposure, a few cell types, mostly T-lymphoma cells, show up-regulation of GR expression in response to ligand. For example, the human leukaemic T-cell lines CEM-C7 (Eisen et al., 1988) and 6TG1.1 (Denton et al., 1993) as well as the human myeloma cell line OPM-2 (Gomi et al., 1990) and the mouse thymoma cell line WEHI-7 (Gruol et al., 1989) show an increase in GR expression after treatment with a GR agonist. It may therefore be the case, that up-regulation of GR in T-cells, which are sensitive to glucocorticoid-induced apoptosis, is a tissue-specific response related to the biological effect of glucocorticoids in regulating T cell homeostasis.

In the adult animal, autoregulatory mechanisms therefore exert potent and dynamic control of GR expression in a tissue-specific manner. However, there is also evidence that the perinatal environment can permanently and specifically 'programme' GR levels; a phenomenon in which this laboratory has a particular

interest (Seckl, 1997; Seckl et al., 1999). In this way, any dynamic regulation of GR that occurs in response to glucocorticoids or other stimuli fluctuates around a set, 'programmed' level.

1.2.2.2.2 Perinatal programming of GR levels

The foetal origins of disease hypothesis states that early life events can permanently 'programme' the development of tissues and organs thus influencing adult physiology and later pathophysiology (Barker, 1990). In particular, maternal nutrition contributing to low foetal birth weight correlates very strongly with the development of hypertension, non-insulin dependent diabetes mellitus and death from ischaemic heart disease in adulthood (Barker et al., 1990; Barker, 1997). Significantly, antenatal glucocorticoid therapy (for example as a treatment for babies at risk of congenital adrenal hyperplasia) results in offspring with reduced birth weight (reviewed in Seckl, 2000). In this laboratory, a rat model of this programming effect was developed in which pregnant females were exposed to the synthetic glucocorticoid dexamethasone in the third week of pregnancy (Nyirenda et al., 1998). The offspring of dexamethasone-treated rats showed reduced birth weight and both fasting and post-glucose hyperglycaemia as adults (Nyirenda et al., 1998). Furthermore, levels of GR and PEPCK were permanently elevated in the periportal region of the liver, which is the major site of hepatic gluconeogenesis (Nyirenda et al., 1998).

Interestingly, while rat hepatic GR levels are increased after dexamethasone exposure *in utero*, hippocampal GR levels are decreased (Holmes et al., 1997). In behavioural tests these animals show impaired learning and increased anxiety, probably due to increased HPA axis activity (Holmes et al, 1997). Furthermore, adult rats exposed to stressful stimuli show permanently decreased GR mRNA levels in several brain regions including the hippocampus and cerebellum (Kitraki et al., 1999). Conversely, a post-natal increase in 'maternal care' up-regulates GR expression in the hippocampus, leading to greater hippocampal sensitivity to glucocorticoids and enhanced negative HPA feedback efficacy (Meaney et al., 1988).

The paradigm for maternal care has been neonatal handling, in which pups are separated from mothers for short (15 minute) intervals during the first three weeks of life. This separation results in altered maternal behaviours upon reuniting, including increased licking and grooming of pups (Liu et al., 2000). Handled animals show blunted ACTH and corticosterone responses to stress and hence more effective shut-down of HPA activity following stress. In addition, the increased hippocampal GR levels present in handled rats are correlated with reduced hippocampal cell loss and improved spatial memory in old age (Meaney et al., 1988). As such, neonatal handling appears to offer protection against the chronic detrimental effects of stress-activation of the HPA axis.

The mechanisms underlying the permanent programming of GR levels by early life events is poorly understood. While work discussed previously in this chapter suggests that GR can induce chronic effects on the DNA methylation status and chromatin conformation of target genes, and that GR levels are subject to autoregulation, the divergent responses of the liver and hippocampus to glucocorticoid exposure *in utero* is still a source of confusion. With regards to the selective up-regulation of hippocampal GR after neonatal handling, a pathway in which thyroxine, 5-HT and cAMP act to up-regulate GR expression has been proposed (Mitchell et al., 1992; Meaney et al., 2000). In addition, chronic environmental enrichment has been shown to up-regulate expression of the (NGF-induced) immediate early gene NGFI-A and GR in rat hippocampus, once again demonstrating that chronic environmental manipulations can alter hippocampal GR expression in adult rats (Olsson et al., 1994).

1.2.2.2.3 Transcriptional regulation of GR

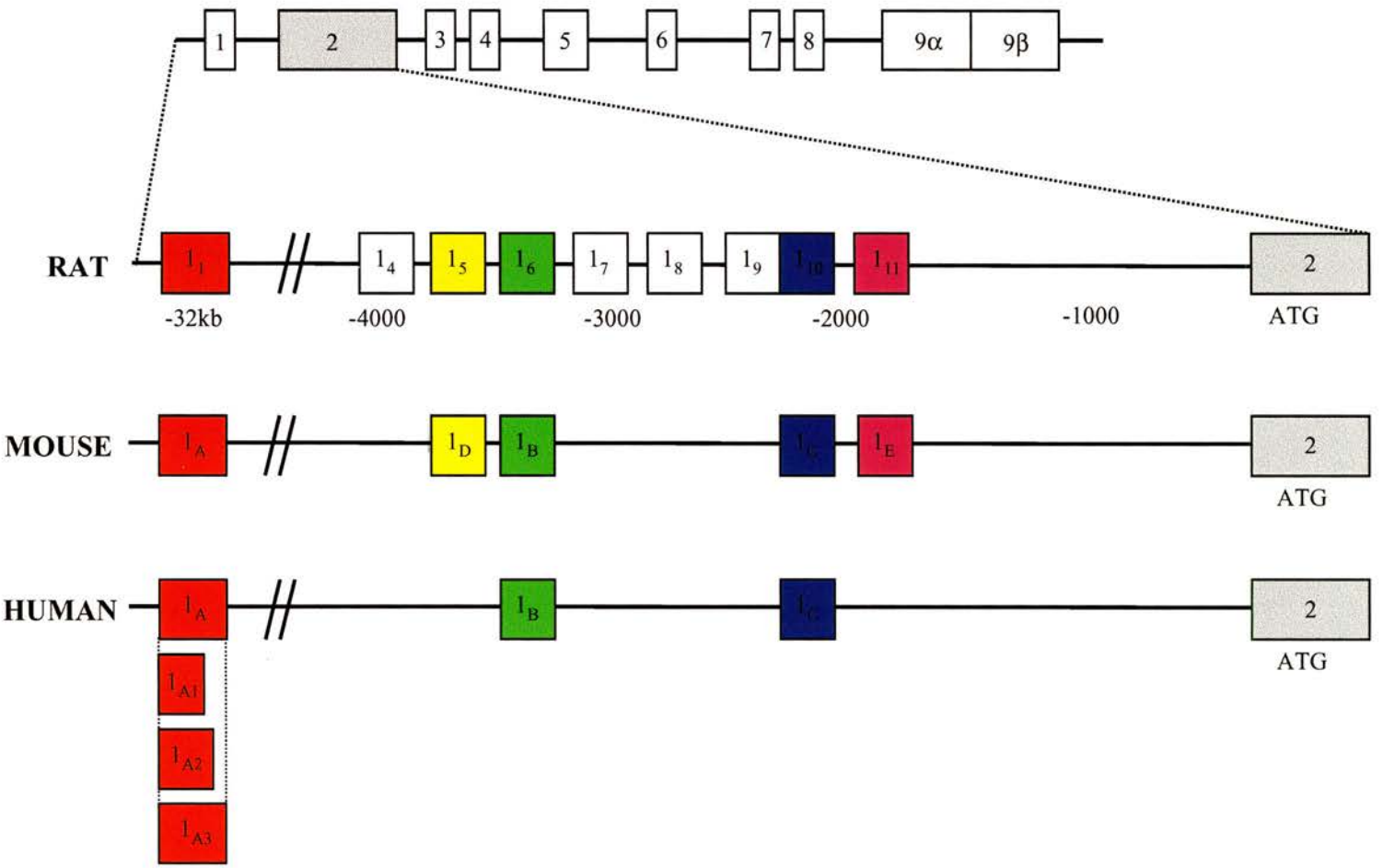
Genetic manipulation in both *in vitro* and *in vivo* studies have therefore demonstrated that maintaining appropriate levels of GR expression is crucial in maintaining normal physiology. Similarly, studies have shown that tissue-specific levels of GR expression are both autoregulated and subject to perinatal programming. Furthermore, while analyses of GR in various tissues have shown it to be

ubiquitously expressed (Ballard et al., 1974), GR levels appear to vary widely between tissues (Funder, 1992). For example, Northern blot analysis of GR mRNA in rat tissues showed that levels were greatest in the lung, with the relative levels in other tissues as follows: spleen, 70%; brain, 55%; liver, 50%; kidney, 43%; heart, 35%; adrenal, 13%; and testis only 8% (Kalinyak et al., 1987). In addition, GR mRNA levels have been shown to vary within tissues, most demonstrably in subfields of the hippocampus (Herman & Spencer, 1998). The question therefore arises of how such complex and tissue-specific regulation of GR levels is achieved.

Investigation into this question has naturally focused on the GR promoter. The structure of the GR promoter has been partially elucidated in the human (Zong et al., 1990; Encio & Detera-Wadleigh, 1991; Govindan et al., 1991), mouse (Strahle et al., 1992; Chen et al., 1999) and rat (Gearing et al., 1993; McCormick et al, 2000). In all species so far investigated, the promoter region of GR contains no obvious TATA or CAAT elements and comprises multiple exons 1 that are alternatively spliced onto a common exon 2. However, an in-frame translational stop codon downstream of the splice acceptor site in exon 2 means that the sequence of the translated protein is unaffected by the first exon. As such, all alternate exon 1 GR transcripts encode a common protein but differ in their 5' leader sequence.

At least five alternate exons 1, termed 1A, 1B, 1C, 1D and 1E, have been described for the mouse GR gene (Strahle et al., 1992; Chen et al., 1999; fig 1.4). Human homologues to 1A, 1B and 1C have also been identified (Nunez & Vedeckis, 2002), with further evidence suggesting that alternative splicing events generate three different hGR 1A-containing transcripts, termed 1A1, 1A2 and 1A3 (Breslin et al., 2001; fig. 1.4). Evidence from this laboratory has demonstrated the existence of at least eleven potential alternate GR exons 1 in the rat; 1_1, 1_2, 1_3, 1_4, 1_5, 1_6, 1_7, 1_8, 1_9, 1_{10} and 1_{11} (McCormick et al., 2000; McCormick, 2000). Sequence analysis has shown that exons 1_1, 1_5, 1_6, 1_{10} and 1_{11} are homologous to mouse and human exons 1A, 1D, 1B, 1C and 1E respectively (McCormick et al., 2000; McCormick, 2000; fig. 1.4). In addition, further data from this laboratory have identified homologues to all rat alternate exons 1 in several human tissues (Reynolds, 2002). Interestingly, in

Figure 1.4 *5'-heterogeneity of GR mRNA is conserved across species.*
Schematic representation of alternate exons 1 identified in rat, mouse and human to date (see text for details and references). Homologous exons are shown in the same colour. The approximate locations of exons 1 are shown relative to the translation start (ATG) in exon 2. The 5' extents of alternate exons 1 show considerable heterogeneity that is not represented in this figure.


1
2
3
4
5
6
7
8
9α
9β
RAT
1 1
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
-32kb
-4000
-3000
-2000
-1000
ATG
MOUSE
1 A
1 D
1 B
1 C
1 E
2
ATG
HUMAN
1 A
1 B
1 C
2
ATG
1 A1
1 A2
1 A3

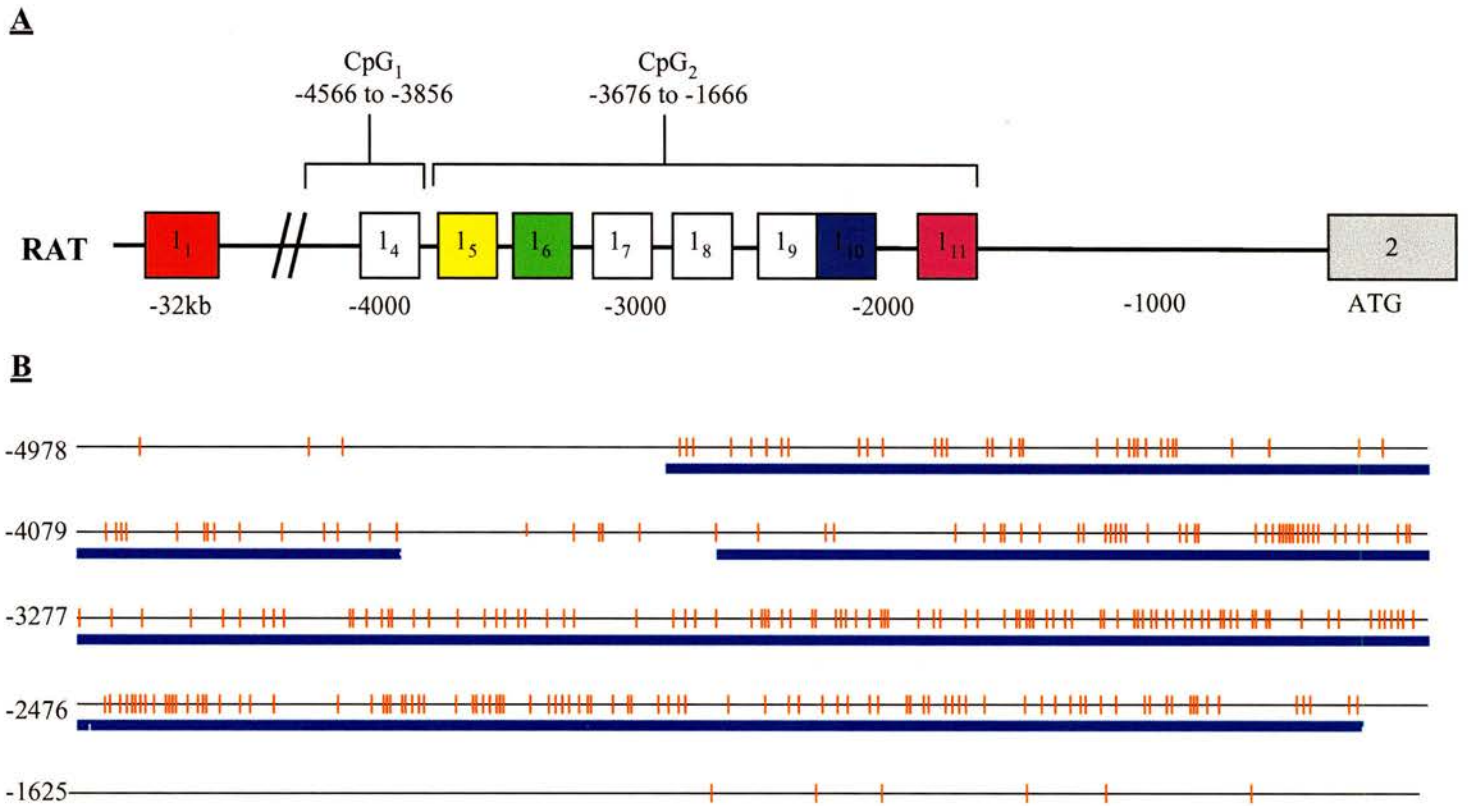
all species so far investigated, most alternate exons 1 are located within ~3.5kb of each other just upstream from exon 2, however, exon 1_1/1A is located ~30.2kb upstream of the translation start site in exon 2 (Strahle et al., 1992; McCormick et al., 2000; McCormick, 2000; Breslin et al., 2001)

1.2.2.2.3.1 The GR promoter occupies a region containing two putative CpG islands.

Analysis of the rat GR promoter with a CpG island predictor program (http://www.ebi.ac.uk/emboss/cpgplot) strongly predicts two CpG islands extending from –4557 to –3847 (CpG_1) and from –3667 to –1651 (CpG_2), relative to the translation start at +1. As such, exon 1_4 lies within CpG_1, while exons 1_5, 1_6, 1_7, 1_8, 1_9 and 1_{10} are located within CpG_2 (fig 1.5). In contrast, exon 1_1, which is located ~30.2kb upstream of the translation start in exon 2, is not located in a CpG island. CpG islands are known to be associated with several characteristics that may have important implications for the transcriptional activity of the GR promoter. CpG islands are stretches of non-methylated DNA rich in CpG dinucleotides, that are found at the promoters of all 'housekeeping' genes and at about half of genes showing tissue-restricted expression patterns (reviewed in Antequera & Bird, 1993). CpG is normally a target for DNA methylation and is under-represented in the genome due to the high mutation rate at 5-methylcytosine (Bird, 1986). The striking immunity of CpG island sequences to methylation has therefore been the subject of considerable investigation.

The current model is based on the association between CpG islands and promoters that also serve as origins of replication (Delgado et al., 1998; Ponger et al., 2001). As such, it is proposed that the transcriptional status of a promoter early in development is 'memorised' by the DNA methylation pattern (Antequera & Bird, 1999). In this way, promoters that are active early in development exclude methylation by recruiting the molecules that initiate DNA replication, a status that is transmitted to all somatic cells by maintenance methylation (Antequera & Bird, 1999).
There is an important relationship between DNA methylation, chromatin

Figure 1.5 *The rat GR promoter contains two CpG islands.*
All locations are given relative to the translation start (ATG) in exon 2.
A: Schematic representation of the location of two CpG islands on the rat GR promoter.
B: Output of a CpG island prediction program, shown to scale (see text for details). CpG dinucleotides are represented by a red vertical line. A blue line shows the extent of the two CpG islands.


A
CpG1
-4566 to -3856
CpG2
-3676 to -1666
RAT
1 1
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
-32kb
-4000
-3000
-2000
-1000
ATG
B
-4978
-4079
-3277
-2476
-1625

modification and gene expression. Experiments have demonstrated that artificially methylated DNA can adopt a distinctive chromatin structure upon integration into the genome that is associated with repression of gene activity (Keshet et al., 1986; Kass et al., 1997). Conversely, unmethylated CpG islands possess a nuclease-sensitive chromatin structure distinct from bulk methylated chromatin and are strongly correlated with transcriptional activity (Tazi & Bird, 1990). Acetylation of chromatin-associated histone proteins appears to be an important factor in maintaining the 'open' chromatin structure at CpG islands; studies have shown that the transcriptional repression exerted by the methyl-CpG binding protein MeCP2 occurs through its interaction with the Sin3/histone deacetylase co-repressor complex (Ng & Bird, 1999).

Thus, the presence of two CpG islands in the GR promoter is consistent with both its activity early in development and its role as a 'housekeeping' gene, as well as predicting that the chromatin in this region is in an 'open' conformation. Analysis of the promoter regions of other members of the steroid hormone receptor family using a CpG island predictor program (http://www.ebi.ac.uk/emboss/cpgplot) shows that they also contain CpG islands of varying sizes. The human oestrogen and progesterone receptor (ER and PR respectively) promoter regions each contain a ~500bp CpG island just upstream from the translation start. The human thyroid receptor β (THβ) also contains a ~1000bp CpG island that lies ~400bp upstream from the ATG codon. Finally, the promoter of the human MR gene is most similar to that of GR, containing two CpG islands which stretch over a ~3.5kb region. Given the aforementioned association between CpG islands and both housekeeping and tissue-specifically expressed genes, perhaps the presence of CpG islands in the promoters of these genes is unsurprising. However, it is interesting that the presence of multiple transcription start sites, encoding multiple alternate exons 1, is also conserved between members of the steroid hormone receptor family.

1.2.2.2.3.2 GR is one of many genes that are transcribed from multiple start sites.

The use of multiple promoters and transcription starts appears to be a frequently used mechanism of exerting complex control of gene expression. Alternative promoter usage that results in alternative leader exons, as exemplified by GR, can be seen to affect gene expression in a variety of ways (reviewed in Ayoubi & Van De Ven, 1996). These include the developmental stage-specific (temporal) expression of a gene, the overall level of expression, the tissue-specific expression, the specific capacity to respond to particular cellular or metabolic conditions, the stability of the mRNA, the translation efficiency of the mRNA and the structure of the amino terminus of the protein encoded by the gene. Many genes containing multiple promoters, including GR, lack consensus TATA or CAAT boxes, a feature that is usually associated with heterogeneous transcription initiation (reviewed in Ayoubi & Van De Ven, 1996).

In several cases, genes contain both a promoter with ubiquitous expression and an alternative promoter with a more restricted spatial or temporal pattern. For example, the human phorphobilinogen deaminase gene (PBGD) possesses an upstream promoter that is active in all tissues, while a second promoter, located 3kb downstream, is active only in erythroid cells (Grandchamp et al., 1987; Chretien et al., 1988). Constitutive expression of the mouse cAMP-dependent protein kinase subunit RIα gene is driven by transcription from two untranslated exons 1, while three further alternate exons 1 show a tissue-specific expression pattern (Barradeau et al., 2000). Similarly, an upstream region of the mouse β1,4-galactosyltransferase gene functions as a housekeeping promoter, while a region adjacent to the translation start site serves as a mammary gland-specific promoter (Rajput et al., 1996). Finally, the human STAT5B gene (a member of the Signal Transducer and Activators of Transcription family) possesses two promoters, one of which is embedded in a CpG island and drives constitutive expression, while the other shows a tissue specific pattern of expression (Ambrosio et al., 2002).

There are also several examples in which regulation of overall levels of gene expression in different tissues is controlled by promoters of different strengths. For example, the α-amylase gene contains a weak (downstream) promoter that is active in liver, while a strong (upstream) promoter is active in the parotid gland, causing a more than 100-fold difference in α-amylase expression between the two tissues (Schibler et al., 1983). There are therefore numerous precedents in which transcription from multiple initiation sites is associated with tissue-specific gene regulation, either through promoters that are 'on' or 'off' in selected tissues, or through promoters that display differential activity between tissues.

There are also several examples whereby alternative promoters are used to regulate gene expression levels according to various extra-cellular signals. For instance, only one promoter of the human SRC gene is regulated by hepatic nuclear factor-1α (Bonham et al., 2000). This has particular relevance for members of the steroid hormone receptor family, which are known to contain alternate promoters that are differentially responsive to hormonal and other stimuli (see below).

Transcription from multiple promoters can also generate multiple mRNAs that can potentially be regulated at the post-transcriptional level. There is substantial evidence showing that transcripts containing long GC-rich leader exons and/or 5' leader sequences with low free energy cannot be translated efficiently (Kozak, 1991). In addition, the presence of short open reading frames (sORFs) within leader sequences have also been shown to profoundly affect translation, whereby differences in initiation efficiency at upstream sORFs lead to differences in translational efficiency between mRNAs (reviewed in Morris & Geballe, 2000). Indeed, recent evidence has shown that translation from a sORF present in mouse GR exon 1A is crucial for GR synthesis, as mutation of its ATG codon completely abolishes GR expression from the downstream translation start (Diba et al., 2001).

It is interesting that, in addition to GR, several other members of the steroid hormone receptor family show evidence of alternate promoter usage. Two forms of human progesterone receptor, hPRA and hPRB, are transcribed from two distinct promoters

that generate overlapping leader sequences (Kastner et al., 1990). Furthermore, transcripts initiating at the upstream promoter A encode an additional translation start codon, giving rise to a protein with an N-terminal extension (Kastner et al., 1990). Importantly, transcription from PR-responsive genes (e.g. human GnRH receptor) has been shown to be differentially activated by the two isoforms (Cheung et al., 2000). Furthermore, the ratio of hPRA to hPRB has been shown to vary widely between human cell lines (Richer et al., 2002) suggesting that tissue-specific regulation of the two isoforms has profound effects on the expression patterns of PR-responsive genes.

A total of six human oestrogen receptor-α (hERα) mRNA isoforms have been identified, with all transcripts encoding a common protein, but differing in their 5' leader sequence (reviewed in Kos et al., 2001). Again, results show a differential pattern of hERα mRNA isoforms in a variety of human tissues and cell types (Flouriot et al., 1998). Similarly, the human mineralocorticoid receptor (hMR) is transcribed from at least two alternate promoters giving rise to three alternate untranslated exons 1; 1α, 1β and 1γ (Kwak et al., 1993; Zennaro et al., 1995; Listwak et al., 1996). While results show that both promoters are glucocorticoid-inducible, only the distal promoter (driving 1β expression) is stimulated by aldosterone in a dose and hMR-dependent manner (Zennaro et al., 1996). Results also show that the three MR mRNA variants are differentially distributed within subfields of the hippocampus (a major aldosterone target tissue) (Kwak et al., 1993), with evidence suggesting that they may be differentially regulated during development according to glucocorticoid exposure (Vazquez et al., 1998).

The thyroid hormone receptor β (THβ) also possesses an interesting genomic structure. In *Xenopus laevis*, two THβ genes each possess at least eight alternatively spliced exons 1 in the 5' leader sequence (Shi et al., 1992). Significantly, while three of the alternate exons 1 are present in all THβ transcripts, the two most distal exons, termed a and b, are not found on the same molecule, suggesting that transcription is driven from at least two distinct promoters (Shi et al., 1992). Furthermore, only transcripts containing exon b are up-regulated following exposure to thyroid

hormone (Shi et al., 1992). At least seven retinoic acid receptor α (RARα) isoforms have been isolated and characterised in the mouse (Leroy et al., 1991). All of these isoforms differ in their 5' leader sequences and, in the case of two variants (mRAR-α1 and mRAR-α2), also in the sequences encoding the N-terminal region (Leroy et al., 1991), which is known to be important for trans-activation by other members of the nuclear receptor superfamily. Interestingly, mRAR-α2 and mRAR-α1, were found to be differentially expressed in mouse adult and foetal tissues and only one isoform, mRAR-α2, could be induced by retinoic acid (Leroy et al., 1991). The mouse RAR-β shows a similar genomic structure, with three isoforms generated from the same gene by differential promoter usage and alternative splicing, which also show varying levels in selected tissues (Zelent et al., 1991).

It is therefore apparent that many members of the steroid hormone receptor family are transcribed from multiple promoters that are differentially regulated in a tissue-specific manner, both during development and in response to external stimuli, particularly their own ligands. In this way, we can hypothesise that the multiple exon 1 structure of the GR promoter is involved in its evidently complex and tissue-specific regulation.

1.2.2.2.3.3 Role of the GR promoter

This hypothesis implies that alternate GR exons 1 are regulated in response to cell-specific factors and external stimuli. Work carried out in this laboratory identified six alternate GR exons 1 expressed in a range of rat tissues (McCormick et al., 2000; McCormick, 2000). Table 1.2 summarises the results of RNase protection assays (RPAs) carried out on rat hippocampus, liver and thymus, using probes complementary to each specific exon 1. In hippocampus, liver and thymus, as well as heart, kidney, lung and testis, transcripts containing exon 1_{10} predominated (McCormick et al., 2000; McCormick, 2000). Exon 1_6 –containing transcripts were also observed in all three tissues. Interestingly, exons 1_5 and 1_7 were both present in hippocampal GR mRNA but were below the limit of detection in liver and thymus. Transcripts containing exon 1_{11} were relatively higher in hippocampus than liver but

were below the limit of detection in thymus. Finally, transcripts containing exon 1_1 were only detected in thymus, representing ~25% of total GR mRNA (McCormick et al., 2000; McCormick, 2000).

Exon	Hippocampus	Liver	Thymus
1_1	0	0	25.6%, 27.1%
1_2	0	0	0
1_4	0	0	0
1_5	8.1%, 7.4%	0	0
1_6	17.0%, 20.7%	10.0±1.3% (6)	21.9%, 19.9%
1_7	8.0%±3.7% (3)	0	0
1_{10}	61.3%, 63.8%	74.8%, 77.5%	52.7%, 58.9%
1_{11}	12.5%, 7.9%	2.2%, 1.7%	0

Table 1.2 *Relative abundance of alternative exon 1-containing GR mRNA in rat hippocampus, liver and thymus.*
Summary of RPAs in which the amount of exon 1 is expressed as a percentage of the total amount of GR mRNA in a particular tissue. Where individual values are shown, the results for each of two independent experiments are given. Where the experiment was carried out more than twice, values shown represent the mean ±SEM (n). Levels of exon 1_4- and 1_2-containing transcripts were below the limits of detection (~1-5% of total GR mRNA) in the three tissues examined. Taken from (McCormick et al., 2000).

RT-PCR studies carried out in a range of mouse tissues have shown that all alternate GR exons 1, including 1A, 1B, 1C, 1D and 1E (homologous to rat exons 1_1, 1_6, 1_{10}, 1_5 and 1_{11} respectively), are ubiquitously expressed (Chen et al., 1999). RPA analysis provided a more quantitative technique and showed that while mouse GR exons 1B and 1C were ubiquitously expressed, exon 1A was only detected in the T lymphocyte lines S49 and WEHI-7 (Strahle et al., 1992). The relative levels of 1B and 1C expression within tissues was not assessed in the study performed by Strahle *et al*, although comparison between tissues showed that levels of both 1B and 1C mRNA were higher in mouse liver than in mouse brain, S49 or WEHI-7 cells (Strahle et al., 1992). More recent RT-PCR studies showed that human GR exons 1B and 1C (1_6 and 1_{10}) were expressed in all human cell lines investigated, including IM-9 (B-cell lymphoma), CEM-C7 (T-cell lymphoblast), Jurkat (T-cell ALL), HL-60 (myeloid leukaemia), HeLa (cervical carcinoma), HepG2 (hepatoma), MCF-7 (breast carcinoma), 786-0 (kidney carcinoma), SJSA (osteosarcoma), H1299 (lung

carcinoma) and WI-38 (normal diploid firbroblasts) (Breslin et al., 2001). Of the three 1A splice variants detected by RT-PCR analysis of human GR, only expression of 1A3 was restricted to cells derived from the immune system (IM-9, CEM-C7, Jurkat and HL-60), while transcripts containing exons 1A1 and 1A2 were identified in all other cell lines (Breslin et al., 2001). Once again, quantitative studies did not assess the relative levels of human GR exon 1B and 1C expression within cell lines (Breslin et al., 2001). However, comparison between cell lines showed that levels of exon 1B expression were not significantly different in IM-9, CEM-C7, Jurkat, or HeLa cells, with slightly higher levels observed in WI-38 relative to IM-9 cells (Breslin et al., 2001). In addition, levels of exon 1C expression in CEM-C7, Jurkat, HeLa and WI-38 were shown to be <50% of those detected in IM-9 cells (Breslin et al., 2001).

The hypothesis that patterns of alternate GR exon 1 expression are regulated in a tissue-specific manner implies that multiple exons 1 are driven by multiple associated promoters that are differentially active between tissues. Results from this laboratory have shown that transiently transfected constructs associated with rat GR exon 1_7 activated a reporter gene in a neural cell-specific manner (McCormick et al., 2000; McCormick, 2000). Other studies have also shown that sequences associated with human exons 1B and/or 1C activate reporter genes in transiently transfected systems in a cell-specific manner (Nobukuni et al., 1995; Breslin et al., 2001; Nunez & Vedeckis, 2002). Specifically, while there was no difference in activity between constructs associated with 1B and 1C in HeLa or Jurkat cells, the promoter activity of 1C was significantly higher than 1B in HepG2 cells (Nunez & Vedeckis, 2002).

With regard to the 1B promoter, footprints have been reported at one GATA, three YY1 and four Sp1 transcription factor binding sites (Breslin & Vedeckis 1998; Nunez & Vedeckis, 2002). A putative NF-κB binding site has also been identified in the 1B promoter, although the functional significance of this remains unresolved (Webster et al., 2001). While EMSAs have not provided evidence of GATA-3 binding to the GATA footprint, experiments have confirmed binding of Sp1 and YY1 at their respective sites (Breslin & Vedeckis, 1998; Nunez & Vedeckis, 2002).

In addition, the relative contribution of these binding sites has been shown to be tissue-dependent. Specifically, deletion of the three YY1 sites has been shown to lead to a decrease in promoter activity in HepG2 and Jurkat cells, but not in HeLa cells (Nunez & Vedeckis, 2002). Conversely, deletion of all four Sp1 sites caused a significant down-regulation in promoter activity in HeLa cells, but not in HepG2 or Jurkat cells (Nunez & Vedeckis, 2002).

With regard to promoter 1C, previous studies have identified five putative Sp1 sites, although their contribution to overall and tissue-specific GR regulation has not been investigated (Breslin & Vedeckis, 1998). In addition, Nobukuni *et al* reported the presence of three footprints on the 1C promoter, one of which was shown to bind AP2 in HepG2 cells (Nobukuni et al., 1995). Significantly, deletion of this AP2 site triggered a decline in promoter activity in HeLa and NIH3T3, but not in HepG2, cells (Nobukuni et al., 1995).

Reporter constructs containing sequences associated with exons 1A1, 1A2 and 1A3 have been shown to be active in a variety of human cell types, with the strongest activity observed in cell lines derived from immune cells (Breslin et al., 2001). This observation, together with the greater expression of GR exon 1A-containing transcripts, in particular exon 1A3, in human T cells has been implicated in the mechanism underlying the immune-specific up-regulation of GR levels in response to glucocorticoids (Breslin et al., 2001). An interferon regulatory factor-binding element has been shown to contribute significantly to basal transcription rate of exon 1A-containing promoter constructs (Breslin et al., 2001). Furthermore, the GR 1A promoter region was shown to contain a footprint resembling a glucocorticoid response element that appears to bind GRβ (Breslin et al., 2001). Interestingly, transcription from GR exon 1A appears to be associated with mGR (Chen et al, 1999), a membrane associated/bound isoform of GR that is more strongly correlated with glucocorticoid-evoked lymphocytolysis than the classical GR (Sackey et al., 1997) (see section 1.2.2.1.4). Evidence gained from mGR-enriched S-49 (mouse lymphoma) cells shows that the cellular presence of mGR is highly correlated with GR transcripts containing exon 1A (Chen et al., 1999). Furthermore, transfection of

mGR-less and glucocorticoid lysis-resistant AtT-20 pituitary and HL-60 leukaemic cells with full-length GR 1A cDNA imparted both mGR expression and glucocorticoid lysis-sensitivity to these cells (Chen et al., 1999).

1.3 Aims

The evidence presented in this chapter shows that glucocorticoids exert profound effects on a variety of physiological systems. Furthermore, cellular responses to glucocorticoids are dependent upon the actions of GR, which exists as several different isoforms. *In vitro* and *in vivo* models show that the appropriate regulation of GR levels in every tissue is crucial in mediating GR action. Furthermore, tissue-specific GR levels are both permanently 'programmed' in response to the perinatal environment and dynamically regulated in adult animals by glucocorticoids themselves. Studies of several other members of the steroid hormone receptor family, in which transcription is differentially driven from multiple promoters in response to tissue-, development- and stimuli-specific factors, suggests that the multiple exon 1 structure of the GR promoter may be involved in the evidently complex regulation of GR levels. As such, the experiments described in this thesis were designed to investigate the role of this alternate exon 1 structure in the tissue-specific, constitutive and auto-regulation of GR.

Previous work carried out in the laboratory showed that, while GR exons 1_6 and 1_{10} were present in all rat tissues examined, exons including 1_5 and 1_7 showed a more tissue-restricted pattern (see section 1.2.2.2.3.3). As such, initial RT-PCR analysis aimed to establish 'model' cell lines, displaying specific patterns of exon 1 expression, which could then be used and manipulated in further experiments. In addition, the expression of multiple alternate exons 1 implies that transcription of GR is driven by a complex arrangement of promoter elements. Transient transfection experiments in two different rat cell lines therefore aimed to elucidate potential promoter elements involved in tissue-specific GR regulation, specifically testing whether alternate exons 1 are driven from individual, cell-specific promoters.

DNaseI footprinting and electrophoretic mobility shift assays were also undertaken in order to identify putative transcription factors involved in GR regulation.

Experiments were also designed with the aim of investigating the role of individual exon 1 splice donor sites in overall GR transcriptional regulation. As such, site-directed mutagenesis was used to abolish selected splice donor sites and any subsequent effects on transcriptional activity were analysed by transient transfection, RT-PCR and sequencing techniques.

Naturally, transcriptional regulation of GR must be considered in the context of the chromatin arrangements over the region. In this way, *DNaseI* hypersensitive site mapping was carried out in order to elucidate any cell-specific differences in the chromatin structure at both specific exons 1 and the whole rat GR promoter locus.

Finally, numerous studies have shown that the most potent regulators of GR expression are glucocorticoids themselves (see section 1.2.2.2.1). The role of the GR promoter, specifically that of individual exons 1, in GR autoregulation was investigated using transiently transfected cells treated with the synthetic glucocorticoid, dexamethasone.

2 Materials & Methods

2.1 Materials

2.1.1 General chemicals

All chemicals were obtained from VWR International Ltd or Sigma-Aldrich Company Ltd., unless otherwise stated. Supplier's addresses are given in Appendix I.

NICK columns Scintillation proximity assay reagent Hybond-C nitrocellulose membrane First Strand cDNA Synthesis Kit Poly(dI-dC).poly(dI-dC)	Amersham Pharmacia Biotech UK Ltd.
Bacto™ Tryptone Bacto™ Yeast Extract Falcon tubes 96-well plates	Becton Dickinson UK Ltd.
Protein assay dye reagent concentrate	Bio-Rad Laboratories Ltd.
SeaKem™ LE agarose	Cambrex Bio Science Wokingham Ltd.
Agar Caesium chloride Low melting point agarose 1 kb DNA ladder Yeast tRNA	Invitrogen
Formamide Loading Buffer Reverse Transcription System *Taq*Bead™ Hot Start Polymerase Beetle Luciferin AP2 recombinant protein	Promega Ltd.
High Pure PCR Product Purification Kit *Taq* DNA polymerase Random Primed Labelling Kit	Roche Diagnostics Ltd.
5ml Rohren tubes 1ml eppendorf tubes 0.5ml eppendorf tubes	Sarstedt Ltd.
Kodak MS-1 autoradiographic film	Sigma-Aldrich Company Ltd.
QuickChange™ XL Site-Directed Mutagenesis Kit	Stratagene Europe
Galacto Light Plus Assay Kit	Tropix Ltd.
Whatman 3MM paper Nebauer Improved Haemocytometer Dounce 1ml all-glass homogeniser	VWR International Ltd.

2.1.2 Miscellaneous equipment

Supplier's addresses are given in Appendix I.

Hyperprocessor GeneQuant RNA/DNA Calculator	Amersham Pharmacia Biotech UK Ltd.
Beckman J2-MC centrifuge Beckman Optima TLX ultracentrifuge	Beckman Coulter (U.K.) Ltd.
Lumat LB9501 Luminometer	Berthold Technologies (U.K.) Ltd.
Agarose gel electrophoresis equipment 583 Gel Drier	Bio-Rad Laboratories Ltd.
Eppendorf Mastercycler	Eppendorf AG.
Ultra-turrax TD Homogeniser	IKA Labortechnik
BRL Model V16 vertical electrophoresis tank	Invitrogen
Heraeus Labofuge 400R	Kendro Laboratory Products plc.
Kodak BioMax STS 45I vertical electrophoresis tank	Kodak Ltd.
Seikosha video printer paper	Qbiogene-Alexis Ltd.
Appligene Imager	Quantum Appligene
Shimadzu spectrophotometer 160A	Shimadzu Europa U.K.
Techne Genius Thermocycler Hybridisation oven HB-1D Hybridisation bottles	Techne

2.1.3 Radiochemicals

Supplier's addresses are given in Appendix I.

[α^{32}P] dCTP 370MBq/ml specific activity	Amersham Pharmacia Biotech UK Ltd.

2.1.4 Enzymes & antibodies

All enzymes were obtained from Promega Ltd., unless otherwise stated. Supplier's addresses are given in Appendix I. All antibodies were obtained from Santa Cruz Ltd.

SstI	Invitrogen
DeoxyribonucleaseI (DNaseI)	Worthington Biochemicals
Proteinase K Taq DNA polymerase	Roche Diagnostics Ltd.
RNaseA	Sigma-Aldrich Company Ltd.

2.1.5 Tissue culture reagents & equipment

Supplier's addresses are given in Appendix I.

Dulbecco's Minimal Essential Medium (DMEM) Fetal Calf Serum (FCS) Horse serum (HS) L-Glutamine Lipofectin Opti-MEM Penicillin/Streptomycin RPMI Trypsin/EDTA in HBSS	Invitrogen
F75 Vented Flasks 60mm Dishes	Greiner Bio-One Ltd.
2ml Cryotubes	Nalgene Labware

2.1.6 Solutions & buffers

DEPC dH_2O	0.5ml DEPC in 500ml ultrapure water. Leave for 1-24 h, autoclave.
DNA Loading Buffer	30% v/v glycerol, 0.25% w/v Orange R
DNaseI Protection Buffer	50% glycerol, 50mM Tris-HCl pH 7.5, 12.5mM $MgCl_2$, 5mM $CaCl_2$, 0.5mM EDTA, 375mM KCl, 20mM Spermidine, 2.5mM DTT
DNaseI Stop Solution	2% SDS, 10mM EDTA (pH 8.0)
DNaseI Footprinting Stop Solution	2M NaCl, 0.1M EDTA (pH 8.0)
Freezing Medium	15% DMSO, 85% FCS
Genomic DNA Extraction Buffer	10mM Tris-HCl pH 8.0, 0.1M EDTA (pH 8.0), 20ug/ml RNase A, 0.5% SDS
GTE	50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA
2x HBS	280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4. pH to exactly 7.05 with 10M NaOH.
Hydrazine Stop Solution	0.3M $NaC_2H_3O_2$, 0.1mM EDTA (pH 8.0), 25µg/ml tRNA
10x Kinase Buffer	0.5M Tris-HCl pH 7.5, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA (pH 8.0)
Luria-Bertoni (LB) Broth	1% w/v bactotryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl. If required, ampicillin was added to a concentration of 100µg/ml after autoclaving.

LB-Agar	LB broth with 1.5% w/v agar. If required, ampicillin was added to a concentration of 100μg/ml after autoclaving and before pouring.
Luciferase Assay Buffer	40mM Tricine, 67mM DTT, 0.2mM EDTA, 2mM $MgCO_3$, 5mM $MgSO_4$, 0.25mM coenzyme A. pH adjusted to 7.8
Lysis Buffer	25mM Tris-HCl pH 7.8, 2mM DTT, 1% Triton X-100, 10% glycerol
10 x MOPS	200mM MOPS acid, 50mM $C_2H_3O_2Na$, 10mM EDTA. pH adjusted to 7.2.
Nuclear Extract Buffer A	10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl. 0.5mM DTT and 0.5mM PMSF added just prior to use.
Nuclear Extract Buffer C	20mM HEPES, 25% v/v glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA. 0.5mM DTT and 0.5mM PMSF added just prior to use.
Nuclear Extract Buffer D	20mM HEPES, 20% v/v glycerol, 0.1M KCl, 0.2mM EDTA. 0.5mM DTT and 0.5mM PMSF added just prior to use.
Nuclei Buffer A	85mM KCl, 10mM Tris-HCl pH 7.6, 5.5% w/v sucrose, 2mM EDTA. 10mM PMSF and 0.5mM spermidine added just prior to use.
Nuclei Buffer B	85mM KCl, 10mM Tris –HCl pH 7.6, 5.5.% w/v sucrose, 2mM EDTA, 0.2% Nonidet P40. 0.5mM spermidine added just prior to use.
Nuclei Buffer C	85mM KCl, 10mM Tris –HCl pH 7.6, 5.5.% w/v sucrose, 1.5mM $CaCl_2$, 3mM $MgCl_2$. 0.5mM spermidine added just prior to use.
1 x PBS	1 x PBS tablet (Sigma) dissolved in 200ml dH_2O to give 10mM phosphate buffer, 2.7mM KCl, 137mM NaCl
Potassium Acetate (5M acetate, 3M potassium)	245.6g $KC_2H_3O_2$ dissolved in 442.5ml dH_2O, 57.5ml glacial acetic acid.
Prehybridisation Buffer	5 x SSC, 0.5% SDS, 5 x Denhardt's Solution (50 x Denhardt's Solution = 5g Ficoll, 5g polyvinylpyrrolidone, 5g BSA to 500ml with dH_20).
RNA Loading Buffer	100μl =15μl ethidium bromide (10mg/ml), 85μl DNA Loading Buffer
Southern Depurination Solution	0.125M HCl
Southern Denaturation Solution	0.5M NaOH, 1.5M NaCl
Southern Neutralisation Solution	1.5M Tris-HCl pH 7.2, 1.5M NaCl, 1mM EDTA
Southern Wash Buffer I	2 x SSC, 0.1% SDS
Southern Wash Buffer II	0.5 x SSC, 0.1% SDS
20 x SSC	3M NaCl, 0.3M sodium citrate. pH adjusted to 7.0 with 10M NaOH.

50 x TAE	121.1g Trizma base dissolved in 421.5ml dH_2O, 50ml 0.5M EDTA, 28.6ml glacial acetic acid
10 x TBE	0.9M Tris, 0.9M Boric Acid, 12.5mM EDTA
TE Buffer	10mM Tris-HCl pH 8.0, 1mM EDTA
TEN Buffer	10mM Tris-HCl pH 8, 1mM EDTA, 50mM NaCl
10 x Universal Restriction Buffer	500mM NaCl, 500mM Tris-HCl pH 8.0, 100mM $MgCl_2$, 60mM β-mercaptoethanol, 1mg/ml BSA

2.1.7 Oligonucleotides

All oligonucleotides were purchased from Oswel DNA service (see Appendix I), unless otherwise stated. Locations of oligonucleotides on GR sequence are given in Appendix II. Numbering is with respect to the translation start +1.

2.1.7.1 Oligonucleotides used in PCR

Name	Sequence	Comment
LUC	5'GCGTATCTCTTCATAG 3'	Luciferase primer
GREx2	5'CATGGACAGTGAAACGGC 3'	Rat GR exon 2 primer
$GR1_1$	5'CTTGCTGGAAGTGTCTGGGATG 3'	Rat GR exon 1_1 primer
$MGR1_1$	5'CTTCGTTAGAGTGTCTGGGAGGAAG 3'	Mouse GR exon 1_1 primer
$GR1_4$	5'TTGCACCCCAAAGCAACACC 3'	Rat GR exon 1_4 primer (-4239 to –4219)
$GR1_5$	5'AAGAGGGTTTTGGATTCG 3'	Rat GR exon 1_5 primer (-3575 to –3557)
$GR1_6$	5'ACCTGGCGGCACGCGA 3'	Rat GR exon 1_6 primer (-3303 to –3287)
$GR1_7$	5'AAAGAAACTCGGTTTCCCTC 3'	Rat GR exon 1_7 primer (-2967 to –2947)
$GR1_{10}$	5'GTTGACTTCCTTCTCCGTGA 3'	Rat GR exon 1_{10} primer (-2321 to –2301)
$GR1_{11}$	5'CGGCCTTATCTGCTAGAAGT 3'	Rat GR exon 1_{11} primer (-1749 to –1729)
GR-1	5'GACCTCTTGAAGGATTTGGAG 3'	Rat exon 2 forward primer
GR-2	5'GCTTACATCTGGTCTCATTCC 3'	Rat exon 3 reverse primer
GAPDH-1	5'GTCGGTGTGAACGGATTTGGCCGT 3'	Rat GAPDH forward primer
GAPDH-2	5'CATGGCCTACATGGCCTCCAAGG 3'	Rat GAPDH reverse primer

2.1.7.2 Oligonucleotides used in site-directed mutagenesis

Name	Sequence	Comment
P2Mut1_6F	5'CGAGTCCCCCCCGGGCTCACAACATGTAT -GCGCTGACCCTCTCC 3'	Rat exon 1_6 mutation forward primer (-3290 to –3246 with GT to AC mutation at –3264)
P2Mut1_6R	5'GGAGAGGGTCAGCGCATACATGTTGTGAG -CCCGGGGGGGACTCG 3'	Rat exon 1_6 mutation reverse primer (complimentary sequence to P2Mut1_6F)
P2Mut1_{10}F	5'GAGCGCGCGGGTGCTGAGACGAGCGGGGG CTGGGCGAG 3'	Rat exon 1_{10} mutation forward primer (-2234 to –2196 with GT to AC mutation at –2216)
P2Mut1_{10}R	5'CTCGCCCAGCCCCCGCTCGTCTCAGCACC -CGCGCGCTC 3'	Rat exon 1_{10} mutation reverse primer (complimentary sequence to P2Mut1_{10}F)
P2Mut1_{11}F	5'GCCGCAGAGAACTCAACAGACCTGGACAC -ATTTCTCCCTTCACC 3'	Rat exon 1_{11} mutation forward primer (-1723 to –1679 with GT to AC mutation at –1704)
P2Mut1_{11}R	5'GGTGAAGGGAGAAATGTGTCCAGGTCTGT -TGAGTTCTCTGCGGC 3'	Rat exon 1_{11} mutation reverse primer (complimentary sequence to P2Mut1_{11}F)

2.1.7.3 Oligonucleotides used in Electrophoretic Mobility Shift Assays

Mutations from consensus transcription factor binding sequences are shown in bold.

Name	Sequence	Comment
O_S	5'ATTCGATCGGGGCGGGGCGAGC 3' 3'TAAGCTAGCCCCGCCCCGCTCG 5'	Oligonucleotide containing consensus Sp1 binding site (Santa Cruz)
O_{MS}	5'ATTCGATCGG**TT**CGGGGCGAGC 3' 3'TAAGCTAGCC**AA**GCCCCGCTCG 5'	Oligonucleotide containing mutant Sp1 binding site (Santa Cruz)
O_A	5'GATCGAACTGACCGCCCGCGGCCCGT 3' 3'CTAGCTTGACTGGCGGGCGCCGGGCA 5'	Oligonucleotide containing consensus AP2 binding site (Santa Cruz)

O_{MA}	5'GATCGAACTGACCGC**TT**GCGGCCCGT 3' 3'CTAGCTTGACTGGCG**AA**CGCCGGGCA 5'	Oligonucleotide containing mutant AP2 binding site (Santa Cruz)
O_E	**5'GGATCCAGCGGGGGCGAGCGGGGGCGA 3'** 3'CCTAGGTCGCCCCCGCTCGCCCCCGCT 5'	Oligonucleotide containing consensus Egr-1 binding site (Santa Cruz)
O_{ME}	5'GGATCCAGC**TA**GGGCGAGCTAGGGCGA 3' 3'CCTAGGTCG**AT**CCCGCTCGATCCCGCT 5'	Oligonucleotide containing mutant Egr-1 binding site (Santa Cruz)
O_F	5'CCCAGGCCTCCCCAGAGGGCGTGTCT 3' 3'GGGTCCGGAGGGGTCTCCCGCACAGA 5'	Oligonucleotide encoding FP1 (see chapter 4)
O_{MF}	5'CCCAGGCC**GAA**CCAGAGGGCGTGTCT 3' 3'GGGTCCGG**CTT**GGTCTCCCGCACAGA 5'	Mutant oligonucleotide encoding FP1 (see chapter 4)

2.1.8 Plasmids & Constructs

pSV2L encodes the firefly luciferase gene driven by a mammalian SV40 promoter (de Wet et al, 1987) and was used as a positive control in transient transfection studies. pCH110 (Amersham Pharmacia) is a β-galactosidase expression plasmid and was used as an internal control for transfection efficiency. pRShGR is a human GR expression plasmid and has been described previously (Giguere et al., 1986). pKC302 was a gift from K.E.Chapman. The rat GR competitor construct used in quantitative RT-PCR analysis was made by J.A. McCormick and has been described previously (Lai et al., 2003).

The following constructs, in which fragments of the rat GR promoter are fused to luciferase reporter gene, were made by J.A. McCormick and have been described previously (McCormick et al., 2000; McCormick, 2000): $P1_5$, $P1_6$, $P1_7$, $P1_{7a}$, $P1_{7b}$ and $P1_{7c}$, $P1_8$, $P1_{9/10}$, $P1_{10}$, $P1_{11}$ and P2. All constructs were based on a modified form of pGL3-Basic (Promega), pGL3-BM (McCormick et al., 2000; McCormick, 2000).

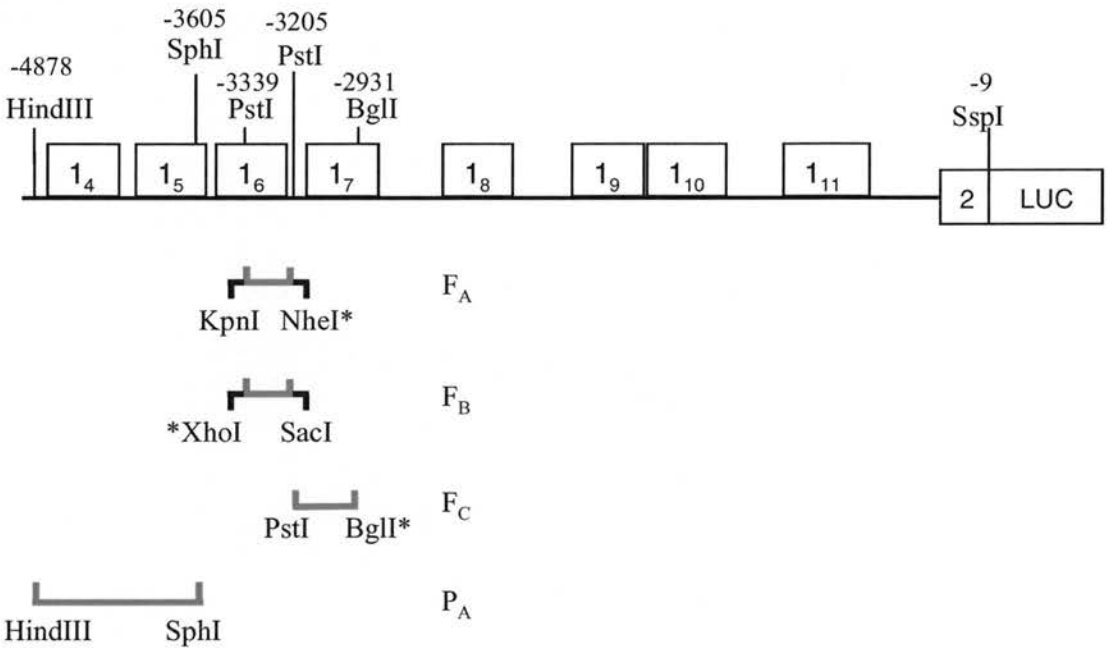
All pVL constructs were a kind gift from V. Lyons. In the case of pVL257, pVL258, pVL259, pVL260, pVL261, pVL262 and pVL263, inserts were excised from P2 and

cloned into pGL3-BM (see above and references therein, in addition to chapter 4). Similarly, a 134bp *PstI* fragment (-3339 to –3205) was isolated from P2 and cloned in the forward (pVL289) and reverse (pVL290) orientations into pGL3-BM. Finally, a *HincII/BglII* fragment containing the 134bp *PstI* fragment was isolated from either pVL289 or pVL290 and cloned in both the forward (pVL287) and reverse (pVL288) orientations into pGL3-Promoter (Promega). To construct $P1_{7bREV}$, a 134bp *PstI* fragment (-3339 to –3205) was isolated from $P1_{7b}$ and re-ligated in the reverse orientation. In pHLM7, a *BglII/KpnI* fragment from –2931 to –2806 was isolated from P2 and cloned into pGL3-BM. $P2Mut1_6$, $P2Mut1_{10}$ and $P2Mut1_{11}$ were synthesised by site-directed mutagenesis (section 2.2.5.4) of P2 using the appropriate primers (section 2.1.7.2). Similarly, $P1_{11}Mut1_{10}$ was synthesised by site-directed mutagenesis of $P1_{11}$.

2.1.9 Probes used in EMSAs and *DNaseI* hypersensitive site mapping

The locations of the labelled fragments used in EMSAs and *DNaseI* hypersensitive site mapping are given in fig. 2.1.

Figure 2.1 *Fragments of the GR promoter used in EMSAs and DNaseI hypersensitive site mapping.*
Diagram showing the position of fragments used in EMSAs and *DNaseI* hypersensitive site mapping and the restriction enzymes used to generate them. ⌜ and ⌝ indicate regions of polylinker DNA derived from pGL3-BM. Recessed 3' ends that are radioactively labelled by the Klenow enzyme are labelled with a '*'. F_{NS}, a 130bp *HindIII/EcoRI* fragment derived from pKC302 is not shown.


-3605
SphI
-3205
PstI
-4878
HindIII
-3339
PstI
-2931
BglI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
KpnI NheI*
F A
*XhoI SacI
F B
PstI BglI*
F C
HindIII SphI
P A

2.2 Methods

2.2.1 Maintenance of Cell Lines

A summary of the maintenance of all cell lines is given in table 2.2.1. All cell lines were maintained in F75 flasks (Greiner) at 37°C and all media were stored at 4°C and equilibrated to room temperature before use. Each cell line was routinely split between 1:3 and 1:10 when confluent. To split / harvest adherent cells including H4IIE, 2S FAZA, KNRK, PC-12 and NG108-15, each flask was washed in 10ml serum free DMEM and treated with 2ml 1 x trypsin for 2 min at 37°C to release the cells. 8ml DMEM with serum was added and mixed gently to give a homogeneous suspension. Cells were then diluted as appropriate in DMEM with serum. B103 cells are adherent but do not require the application of trypsin for release. B103 flasks were given a sharp tap to dislodge the cells and diluted as appropriate with DMEM plus serum. EL4 and S49 cells grow in suspension and were simply diluted as appropriate with DMEM or RPMI plus serum.

For storage, stocks of cells were frozen and kept under liquid nitrogen. Cells from a single confluent flask were resuspended in 3ml freezing medium and transferred in 1.5ml aliquots to cryotubes. The tubes were sandwiched in polystyrene, wrapped in foil and places at –80°C overnight before being transferred to liquid nitrogen.

2.2.2 Gel electrophoresis

2.2.2.1 Analytical agarose gels

DNA was analysed using standard agarose gels of 0.8-2%, prepared as described in Sambrook *et al* (Sambrook et al., 1989). Agarose was dissolved in 0.5 x TBE buffer by boiling in a microwave oven, with higher percentage agarose gels used to resolve smaller DNA fragments. The fluorescent dye ethidium bromide was used to detect DNA and was added to the agarose solution to a final concentration of 0.5ug/ml. The

Cell line	Animal	Cell type	Medium	Serum	Antibiotic	Glutamine	CO_2	Reference
H4IIE	Rat	Hepatoma	DMEM	10% FCS	100µg/ml pen/strep	2mM L-glutamine	5%	(Pitot et al., 1964)
B103	Rat	Neuroblastoma	DMEM	10% FCS	100µg/ml pen/strep	2mM L-glutamine	5%	(Schubert et al., 1974)
2S FAZA	Rat	Hepatoma	DMEM	10% FCS	100µg/ml pen/strep	2mM L-glutamine	5%	(Voice et al., 1996) (Suzuki et al., 1998)
KNRK	Rat	Kidney	DMEM	10% FCS	100µg/ml pen/strep	2mM L-glutamine	5%	(Suzuki et al., 1998)
NG108-15	Rat x mouse hyrbid	Glioma	DMEM	10% FCS	100µg/ml pen/strep	2mM L-glutamine	5%	(Fukuda et al., 1988)
S49	Mouse	Lymphoma	DMEM	10% HS	100µg/ml pen/strep	2mM L-glutamine	5%	(Steinberg et al., 1977)
EL4	Mouse	Lymphoma	DMEM	5% FCS	100µg/ml pen/strep	2mM L-glutamine	10%	(Flaherty & Rinchik. 1978) (Gays et al., 2000)
PC-12	Rat	Adrenal pheochromoctyoma	RPMI	10% HS	100µg/ml pen/strep	2mM L-glutamine	5%	(Levi et al., 1980)

Table 2.1: Maintenance of cell lines summary table.
Abbreviations: DMEM = Dulbecco's Minimal Essential Medium; FCS = Foetal Calf Serum; HS = Horse Serum; pen = penicillin; strep = streptomycin.
Media suppliers are given in section 2.1.5.

warm solution was then poured into a gel mould including the appropriate comb (Bio-Rad), and allowed to solidify. The solidified gel was submerged in a gel electrophoresis tank (Bio-Rad) containing 0.5 x TBE buffer and the comb was removed to create loading wells for the DNA samples. In order to load DNA samples more easily and observe their progress during electrophoresis, DNA loading buffer containing glycerol and Orange R dye was added to each DNA sample (1ul loading buffer per 10µl sample). Electrophoresis was carried out at 70-120V until the dye front had run two thirds down the gel. DNA was visualised on an UV transilluminator at 254nm, imaged using an Appligene Imager and printed on Seikosha video printer paper. DNA fragment size was estimated by comparison to 1kb DNA ladder (Invitrogen).

2.2.2.2 Preparative agarose gels

To gel purify DNA fragments (e.g. for templates in radiolabelling or construction of plasmids) the above procedure was followed with the substitution of 'low melting point' agarose (Invitrogen). Samples were electrophoresed at 80V until the desired fragment was resolved. DNA was visualised on an UV transilluminator at 365nm to minimise UV damage to DNA. The required band was excised with a clean scalpel and purified as described in section 2.2.4.5.

2.2.2.3 Agarose gels for analysis by Southern blotting

0.8% agarose gels for Southern analysis were prepared as described above using 0.5 x TBE. Electrophoresis was carried out in 0.5 x TBE at 80-100V until the dye front had reached the end of the gel. The gel was then treated as described in section 2.2.4.11.

2.2.2.4 Denaturing agarose RNA gels

Integrity of total RNA was verified by denaturing agarose gel electrophoresis. Gel trays, tanks and combs were scrubbed with detergent, soaked in 10M NaOH for 30

min and rinsed with ultra-pure water before use. For a 100ml 1.2% agarose gel, 1.2g agarose was dissolved in 88ml DEPC water by boiling in a microwave oven. The solution was allowed to cool in a fume hood before 2ml formaldehyde and 10ml 10 x MOPS were added and the gel was poured. The solidified gel was submerged in a gel tank containing 1 x MOPS for 15 min prior to sample loading. Typically, 5µg of sample RNA was used, the volume of which was adjusted to 10µl with DEPC water. Each RNA sample was denatured by adding 2.5µl formaldehyde, 2.5µl 10 x MOPS and 10µl deionised formamide in a fume hood, before incubation at 65°C for 15 min. 2.5µl RNA loading buffer (including ethidium bromide) was added to each of the denatured samples before loading onto the gel and electrophoresis at 100V. RNA was visualised on an UV transilluminator at 254nm and imaged as described previously.

2.2.2.5 Denaturing polyacrylamide gel electrophoresis

DNaseI footprinting reactions were run on vertical denaturing polyacrylamide gels. Glass plates were scrubbed with detergent, rinsed with water and wiped with 100% ethanol prior to use. One side of one glass plate was siliconised by wiping approximately 1.5ml Sigmacote over the surface. For a 10% denaturing gel 0.3mm spacers were sandwiched between two glass plates (45cm x 35cm), clamped with bulldog clips and sealed with 1.5% agarose (w/v in dH_2O). 42g urea was dissolved in 25ml 40% acrylamide:bis-acrylamide (19:1), 10ml 10 x TBE, 600µl freshly prepared 10% ammonium persulphate and made up to 100ml with dH_2O. Polymerisation was started by the addition of 125µl TEMED to the mix, which was then immediately poured into the gel cast. The comb was inserted quickly and the gel allowed to polymerise for at least 2h before assembly into the electrophoresis apparatus (Kodak BioMax STS 45i). The gel was pre-run at 1500V (using a Bio-Rad Power Pac 3000) for at least 30 min. Samples (containing formamide loading buffer) were heated at 100°C for 10 min prior to loading. Care was taken to flush out wells with buffer immediately prior to loading, in order to remove any urea. Electrophoresis was carried out at 1600 – 1800V (not exceeding 40mA) until the bromophenol blue present in the loading buffer reached the bottom of the gel. For enhanced resolution

of large DNA fragments (>100bp), a second loading of sample was applied and again electrophoresed until the bromophenol blue reached the bottom of the gel. The gel was then transferred to Whatman 3MM paper, covered with Saranwrap and dried under vacuum at 80°C for 2h on a BioRad 583 Gel Drier. DNA was visualised as described in 2.2.3.

2.2.2.6 Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was used in gel mobility shift analysis. As above, glass plates were scrubbed with detergent, rinsed and wiped with 100% ethanol prior to use. However, neither plate was siliconised. 1.5mm spacers were sandwiched between two glass plates (17cm x 15cm), clamped with bulldog clips and sealed with 1.5% agarose (w/v in dH_2O). A gel mix containing 2.5ml 10 x TBE, 6.25ml 40% acrylamide:bis-acrylamide (19:1) (for 5% gels) and 300µl fresh 10% ammonium persulphate was made up to 50ml with dH_2O and polymerisation was initiated with 40µl TEMED. The mix was immediately poured into the gel cast and a 20 tooth comb inserted. The gel was allowed to set for at least 2h and pre-run at 100V for 30 min in 0.5 x TBE on a BRL model V16 apparatus. Samples were loaded without loading dye (lanes were numbered) and electrophoresed for approximately 90 min at 200V. DNA loading dye was loaded into empty lanes to check sample progression. Following electrophoresis, gels were transferred to Whatman 3MM paper, covered with Saranwrap and dried under vacuum at 80°C for 1h on a Bio-Rad 583 gel drier. Protein-DNA complexes were visualised as described in 2.2.3.

2.2.3 Autoradiography

Southern blots and dried gels containing ^{32}P were exposed to either a Fujifilm phosphorimager screen and processed on a Fujifilm FLA-2000 phosphorimager and/or to Kodak BioMax MS-1 (maximum sensitivity) film at -80°C inside cassettes containing two intensifying screens, which were then developed using an Amersham Hyperprocessor.

2.2.4 DNA and RNA techniques

2.2.4.1 RNA isolation by TRIzol extraction

RNA was isolated from frozen tissue, monolayer cells and suspension cells using TRIzol reagent (Invitrogen). 50-100mg frozen tissue was homogenised in 1ml TRIzol reagent in pre-chilled eppendorf tubes using an Ultra-turrax TB homogeniser (IKA Lobortechnik). Monolayer cells were washed twice in 1 x PBS before TRIzol was applied at 0.1ml/cm^2. Suspension cells were spun at 1200rpm (Heraeus Labofuge 400R) for 3 min at 4°C, resuspended in 10ml 1 x PBS and counted in a Nebauer Improved Haemocytometer. TRIzol was added at 1ml/5-10 x 10^6 cells.

TRIzol solutions from all three sources were then treated with 0.2ml chloroform per 1ml TRIzol, vortexed vigorously for at least 15s and incubated at room temperature for 3 min. Solutions were spun at 12000g for 15 min at 4°C and the upper aqueous layer transferred into fresh pre-chilled eppendorf tubes. Isopropanol was added at 0.5ml per 1ml TRIzol and the tubes inverted gently, before incubation at room temperature for 5 min. RNA was precipitated by centrifugation at 12000g for 10 min at 4°C. Pellets were washed in 75% ethanol and spun at 7500g for 5 min at 4°C. Supernatants were removed and pellets were air-dried at room temperature for 5 min. RNA was resuspended in 20-50µl DEPC dH_2O, its concentration measured using a GeneQuant spectrophotometer (Pharmacia Biotech), and stored at –80°C.

2.2.4.2 Genomic DNA isolation

Isolation of genomic DNA from monolayer cells (B103 and H4IIE) was carried out according to Blin & Stafford (Blin & Stafford, 1976). Cells were harvested from F75 flasks as described in section 2.2.1 and transferred to a pre-chilled 50ml falcon tube. After centrifugation at 1200rpm for 5 min at 4°C (Heraeus Labofuge 400R), cells were resuspended in 5ml cold 1 x PBS and counted using a Neubauer Improved Haemocytometer. After a further centrifugation at 1200rpm for 5 min at 4°C, cells were resuspended in TE to give a concentration of 5 x 10^7 cells/ml. The solution was

then transferred to an appropriate conical flask (50ml flask for 1ml cell suspension, 100ml conical flask for 2ml cell suspension etc.) and 10ml Genomic DNA Extraction Buffer was added per ml of cell suspension. The solution was incubated at 37°C for 1h, before the addition of proteinase K (20mg/ml) to a final concentration of 100µg/ml. The suspension of lysed cells was then incubated in a water bath at 50°C for 3h, swirling periodically. After cooling the solution to room temperature and transferring it to a 50ml falcon tube, an equal volume of phenol/chloroform/IAA (pH8.0) was added and gently mixed by slowly inverting the tube end over end for 10 min. The resulting emulsion was spun at 4300rpm for 15 min at room temperature (Heraeus Labofuge 400R) to separate the two phases. The viscous aqueous phase was carefully transferred with a wide-bore pipette (0.3cm diameter) into a fresh falcon tube and the phenol/chloroform/IAA extraction repeated twice. After the third extraction, 0.2 vol 10M ammonium acetate and 2 vol 100% ethanol were added and swirled until the solution was thoroughly mixed. The resulting precipitate was collected by centrifugation at 4300rpm for 5 min at room temperature and washed twice in 70% ethanol. The pellet was air-dried and resuspended in 1ml TE/5 x 10^6 cells. Complete resuspension required 12-24h of gentle agitation of the DNA solution on a rotating platform at room temperature. DNA concentration was measured using a GeneQuant spectrophotometer (Pharmacia Biotech) and samples were stored at –20°C.

2.2.4.3 DNA purification

When necessary, DNA from restriction digests, *DNaseI* digests and *DNaseI* footprinting reactions was purified by phenol/chloroform extraction and ethanol precipitation as follows. Starting with DNA solution in an eppendorf tube, an equal amount of phenol/chloroform/IAA was added, followed by vigorous vortexing and centrifugation at 12000g for 2 min at room temperature. The upper aqueous layer was transferred to a fresh eppendorf tube and an equal amount of chloroform was added. After vigorous vortexing and centrifugation at 12000g for 2 min at room temperature, 1/20 vol 3M sodium acetate and 2 vol 100% ethanol were added. DNA was precipitated by incubation for no more than 7 min on dry ice followed by

centrifugation at 12000g for 10 min at room temperature. Pellets were washed in 70% ethanol, air-dried and resuspended as appropriate in dH_2O, TE or loading buffer.

2.2.4.4 Restriction Digests

Single restriction enzyme digests were typically performed in a total reaction volume of 20µl, containing 2µl 10 x restriction enzyme buffer, 1µl restriction enzyme (2-10U) and 0.5 – 15µg plasmid DNA (made up to 20µl with dH_2O). Reactions were incubated at optimum temperature for 1-4h. Strategies for double restriction enzyme digests depended upon enzyme buffer compatibility. If both restriction enzymes worked optimally in the same buffer then a standard digest as described above, incorporating 1µl (2-10U) of a second enzyme, was carried out. If no compatible buffer could be used, the digests were performed sequentially. The enzyme with the lower salt buffer was used in the first digest, and inactivated by heating at 65°C for 15 min. Sufficient salt to achieve the concentration required for the second digest was then combined into the reaction and the second restriction enzyme was added. Reactions were run on either analytical or preparative agarose gels after the addition of DNA loading buffer (1µl loading buffer per 10µl sample).

2.2.4.5 DNA Fragment Recovery

DNA fragments required for construction of plasmids or templates for radioactive probes were run on preparative agarose gels as described in section 2.2.2.2. Excised bands were purified using a Roche High Pure PCR Product Purification Kit according to the manufacturer's instructions. Samples were eluted in 15-30µl dH_2O and stored at –20°C until required.

2.2.4.6 Ligation of Plasmid Vector and Insert DNA

The concentration of both vector and insert DNA was estimated by comparison with known DNA concentrations during agarose gel electrophoresis. Ligation reactions

were prepared in a total volume of 10µl, containing 1U T4 DNA ligase (Promega), 1µl 10 x ligation buffer and vector and insert DNA at amounts corresponding to the appropriate molar ratio (usually 1:1). Reactions were incubated at 10°C overnight and subsequently stored at 4°C.

2.2.4.7 Preparation of ^{32}P-Labelled DNA Fragments for Southern Analysis

Probes for Southern analysis were generated using the Klenow fragment of *DNA polymerase I* using a Roche Random Primed Labelling Kit. Specifically, ~50ng purified DNA fragment (made up to a volume of 9µl with dH_2O) was denatured by heating for 10 min in a glass beaker of boiling water, then plunged immediately into ice for 3 min. After a pulse spin, 2µl Solution 6 (random hexamers and buffer), 3µl unlabelled nucleotides (dGTP,dATP,dTTP), 5µl [α^{32}P]dCTP (~3000Ci/mmol) and 10U Klenow fragment (Promega) were added and the mixture was incubated for 1h at 30°C.

Unincorporated label was removed by passing the probe through a NICK column (Amersham). NICK columns were prepared by removing storage buffer and rinsing with 1ml TE prior to use. A further 3ml TE was allowed to drain through the column before the probe was added. The probe volume was made up to 30µl with TE buffer and added to the column. Two 400µl aliquots of TE buffer were then added. The eluate of the first aliquot was discarded while that of the second was retained. 1µl of the second eluate was counted in 1ml liquid scintillant using a scintillation counter. Specific activity was approximately 3500cpm/pg (assuming 70% recovery through NICK column). Probes were denatured by heating to 100°C for 5-10 min before addition to the hybridisation (see section 2.2.4.11)

2.2.4.8 Preparation of ^{32}P-Labelled DNA Fragments for *DNaseI* Footprinting and Gel Mobility Shift Assays

Probes for *DNaseI* footprinting reactions and gel mobility shift assays were generated by 3'-end labelling using the Klenow fragment of *DNA polymerase I.*

Probes were synthesised such that only one end of the fragment incorporated the label (i.e. there was only one recessed 3' end on which Klenow could act). Reactions were carried out in a total volume of 25µl, incorporating 300ng purified DNA fragment (made up to 14.5µl with dH_2O), 2.5µl 10 x Universal Restriction Buffer, 2µl unlabelled nucleotides (dGTP,dATP,dTTP) (Amersham), 5µl [α^{32}P]dCTP(~3000Ci/mmol) and 1µl (10U) Klenow fragment (Promega). Reactions were incubated at room temperature for 10 min and the enzyme inactivated by a further incubation at 60°C for 10 min. Unincorporated label was removed using a Roche High Pure PCR Product Purification Kit. Specifically, 300µl Binding Buffer was added to the probe, the mixture was transferred to a Purification Column and centrifuged at 12000g for 1 min. 500µl Wash Buffer was then added to the column and centrifuged at 12000g for 1 min. This step was repeated with 200µl Wash Buffer. Finally, the probe was eluted in 50µl dH_2O and 1µl was counted in 1ml liquid scintillant as described previously. Specific activity was approximately 40000cpm/pmol fragment.

2.2.4.9 Preparation of ^{32}P-Labelled Double-Stranded Oligonucleotides for Gel Mobility Shift Assays

Pairs of complimentary single stranded oligonucleotides were diluted to 50pmol/µl in TEN buffer and annealed by heating to 90°C for 10 min, followed by cooling slowly to room temperature overnight. Complimentary oligonucleotides were labelled using T4 DNA polynucleotide kinase in a 20µl reaction containing 100pmol complimentary oligonucleotides, 2µl 10 x Kinase Buffer, 2ul [γ-^{32}P]ATP (~3000Ci/mmol) and 1µl (5U) T4 polynucleotide kinase (Promega). Reactions were incubated at 37°C for 1h, before the enzyme was inactivated by the addition of 80µl TE and incubation at 68°C for 10min. Unincorporated label was removed using a Roche High Pure PCR Product Purification Kit as described in section 2.2.4.8. Labelled oligonucleotides were eluted in 50µl dH_20 to give 1pmol/µl (assuming a 50% recovery from purification through the column) and counted using a

scintillation counter. Specific activity was approximately 5000cpm/pmol double-stranded oligonucleotides.

2.2.4.10 Maxam & Gilbert Sequencing Reactions

'G + A' Maxam & Gilbert sequencing reactions were carried out in conjunction with *DNaseI* footprinting experiments. The guanine and adenine bases of a radiolabelled DNA fragment were modified with formic acid before cleavage with piperidine and resolution on a denaturing polyacrylamide gel. Typically, 5µl labelled fragment was incubated with 25µl formic acid for between 20s and 4 min and the reaction stopped with 200µl 'hydrazine stop' buffer. DNA was precipitated by treatment with 250µl 0.3M sodium acetate and 750µl 100% ethanol before incubation on dry ice for no more than 5 min. Following centrifugation for 5 min at 12000g, samples were washed with 100% ethanol and air-dried at room temperature for at least 5 min. Pellets were resuspended in 75µl 1M piperidine and incubated at 90°C for 20-30 min. Samples were then precipitated by addition of 1µl tRNA (10µg/µl) and 400µl 100% ethanol for 5 min on dry ice. After centrifugation for 5min at 12000g, samples were again washed with 100% ethanol and air-dried at room temperature for at least 5 min. Finally, samples were resuspended in 4µl formamide loading buffer (Promega) and heated at 100°C for 5-10 min before loading onto the gel.

2.2.4.11 Southern Blotting & Hybridisation

Southern analysis was carried out on restriction digests of genomic DNA and on *DNaseI* treated DNA as part of *DNaseI* hypersensitive site mapping (see section 2.2.10.2). For Southern analysis of genomic DNA from B103 and H4IIE cells, 15µg DNA (see section 2.2.4.2) was digested with the appropriate restriction enzyme (see section 2.2.4.4). Restriction digested and *DNaseI* treated DNA samples were purified according to section 2.2.4.3, resuspended in 20µl dH_2O and run on Southern agarose gels (see section 2.2.2.3). Following electrophoresis, Southern gels were rinsed in dH_2O and the DNA depurinated by soaking in Southern Depurination Solution with gentle agitation on a rotating platform for 10 min. The gel was again rinsed in dH_2O

and the DNA denatured by soaking in Southern Denaturation Solution with gentle agitation for 30 min. After a final rinse in dH_2O, DNA was neutralised by soaking in Southern Neutralisation Solution with gentle agitation for 20 min. This neutralisation step was repeated. DNA was blotted using a wick of 2 sheets of Whatman 3MM paper in a tray of 20 x SSC. The wicks were flooded with 20 x SSC before the gel was carefully placed on top ensuring no bubbles were trapped between the layers. The gel was flooded with 20 x SSC and a pre-trimmed piece of Hybond-C nitrocellulose membrane (Amersham Pharmacia) was carefully placed on top, again ensuring no bubbles were present. Three sheets of Whatman 3MM paper, pre-cut to size and pre-wet in 20 x SSC were then laid on top of the membrane, and the rest of the wick was masked off with Saranwrap and parafilm to prevent 'short-circuiting'. Finally, a stack of absorbent paper towels were placed on top of the Whatman paper and the apparatus left overnight. Following transfer, membranes were baked between 2 sheets of Whatman 3MM paper for 2h at 80°C.

Prior to hybridisation, membranes were rehydrated in 5 x SSC at room temperature. Subsequently, membranes were pre-hybridised in pre-warmed Prehybridisation Buffer for at least 30 min at 65°C in tubes in a hybridisation oven. Denatured probe (see section 2.2.4.7) was then added to the tube and hybridised for at least 16h at 65°C. After hybridisation, membranes were washed in approximately 50ml Southern Wash Buffer I for 5 min at 65°C. Two further 10 min washes in Southern Wash Buffer I followed. If required, membranes were subsequently washed for 10 min in Southern Wash Buffer II, repeated as necessary. Membranes were wrapped in Saranwrap to prevent them drying out and exposed against autoradiographic film as described in section 2.2.3.

2.2.5 PCR Based Techniques

2.2.5.1 Exon 1 Specific RT-PCR

Reverse transcription of RNA samples was carried out using the Promega Reverse Transcription System. Reactions were prepared using 4μg RNA and 1μg of either

oligo$(dT)_{15}$ or random primers in a total volume of 40µl according to the supplied protocol. For each RNA sample, reactions both with and without AMV Reverse Transcriptase were prepared in order to control for DNA contamination in the subsequent PCR. RT products were either stored at –20°C or used immediately as substrates for PCR.

PCR reactions were carried out using a Promega *Taq*Bead Hot Start Polymerase kit according to the manufacturer's instructions. Reactions included 5µl RT product and 240pmol of each 3'(exon 2/luciferase) and 5' (exon 1-specific) primer in a total of 50µl. PCR was performed on an Techne Genius Thermocycler machine according to the following regime (each reaction was overlaid with 2 drops of mineral oil in lieu of a heated lid):

Segment	Cycles	Temperature	Time
1	1	95°C	4min 10s
2	34	95°C	50s
		50°C	45s
		72°C	1min 30s
3	1	72°C	5min
4	1	**4°C**	Hold

10µl of each reaction was run on an analytical agarose gel.

2.2.5.2 General RT-PCR

RT-PCR reactions were carried out as described in section 2.2.5.1, using GR or GAPDH primer pairs as necessary.

2.2.5.3 Competitive RT-PCR

First strand cDNA synthesis for competitive RT-PCR was carried out using the Amersham Pharmacia First Strand cDNA Synthesis kit. Either 150ng or 200ng sample RNA was incubated with the appropriate concentration of competitor RNA in a total volume of 8µl (made up with nuclease free water) at 65°C for 10min and then

immediately placed on ice. 5µl RT Buffer Mix, 1µl DTT and 1µl Random Hexamers were added to each sample and the mixture incubated at 37°C for 1h. cDNA product was either stored at –20°C or used immediately for PCR.

PCR was carried out in a total reaction volume of 50µl incorporating 32µl nuclease free H_2O, 5µl 10 x *Taq* DNA polymerase buffer + 1.5mM $MgCl_2$ (Roche), 1µl dNTP mix (10mM, Promega), 2µl each of GR-1 primer (10µM) and GR-2 primer (10µM), 7.5µl RT product and 0.5µl *Taq* DNA polymerase (1U/µl, Roche). PCR was performed on a Techne Genius machine according to the following regime (a heated lid negated the need for mineral oil):

Segment	Cycles	Temperature	Time
1	1	94°C	3min
2	30	94°C	40s
		54°C	40s
		72°C	40s
3	1	72°C	10min
4	1	4°C	Hold

2.2.5.3.1 Quantification of Competitive RT-PCR

10µl of each reaction was run on a standard agarose gel and analysed on a Fugi FLA-2000 V1.0 phosphorimager using a fluorescence 473nm:O580 filter. Quantification and comparison of DNA band intensity was carried out using AIDA version 2.0 2D Densitometry software. Data were transferred to an Excel spreadsheet (Microsoft) and absolute levels of sample RNA were calculated by regression analysis as described previously (Lai, 2003).

2.2.5.4 *In vitro* site-directed mutagenesis

Site-directed mutagenesis of plasmid DNA was carried out using the Stratagene QuikChange XL Site-Directed Mutagenesis Kit. Details of plasmid templates and primers employed are given in sections 2.1.7.2 and 2.1.8. Each reaction was carried out in a total volume of 50µl incorporating 5µl 10 x reaction buffer, 5µl plasmid

template (10ng/μl), 1.25μl each primer (100ng/μl), 1μl dNTP (10mM), 2μl QuikSolution, 33.5μl dH_2O and 1μl *PfuTurbo* DNA polymerase (2.5U/μl). Thermal cycling was carried out on a Techne Genius machine according to the following regime (a heated lid negated the need for mineral oil):

Segment	Cycles	Temperature	Time
1	1	95°C	1min 30s
2	18	95°C	1min
		68°C	20min
3	1	68C	7min
4	1	4°C	Hold

Following thermal cycling, each reaction was incubated with 1μl *DpnI* restriction enzyme (10U/μl) at 37°C for 1h in order to digest the parental (nonmutated) DNA.

2.2.6 Cloning of DNA

2.2.6.1 Preparation of competent *E.coli*

HB101, a *RecA* strain of *E.coli*, was used for general cloning procedures. Competent cells were prepared by inoculating 2ml LB with a single HB101 colony and incubating at 37°C overnight in an orbital shaker (160rpm). A further 50ml LB was then added to the culture and incubated at 37°C in an orbital shaker at 160rpm for 1.5-2h (until $A_{600} \approx 0.4$). After pouring into a pre-chilled sterile 50ml centrifuge tube, the cells were collected using a Beckman JA-20 rotor in a Beckman J2-MC centrifuge at 6000rpm for 5 min at 4°C. The resultant pellet was carefully resuspended in 20ml cold 0.1M $CaCl_2$ and incubated on ice for 0.5-2h. Cells were again centrifuged at 6000rpm for 5 min at 4°C and resuspended in 2ml cold 0.1M $CaCl_2$. The resultant competent cells were either used immediately or stored on ice at 4°C for up to 3 days (with daily changes of ice). Transformation of products of site-directed mutagenesis (see section 2.2.6.2) was carried out in XL-Gold ultracompetent cells (Stratagene).

2.2.6.2 Transformations

To transform competent HB101 cells, 200µl cells were pipetted into pre-chilled eppendorf tubes on ice. 200-500ng DNA was added to the cells followed by incubation on ice for 20-30 min. Cells were then heat-shocked at 42°C for exactly 50s and replaced on ice for 2-3 min. The whole reaction was then spread onto LB/Agar/Ampicillin plates and left to air dry for approximately 10 min. Plates were then incubated upside down overnight at 37°C.

Transformation of XL-Gold ultracompetent cells was carried out using the QuikChange XL-Site-Directed Mutagenesis Kit (Stratagene) following the protocol supplied. One tube of cells was thawed on ice and 45µl aliquots were placed in pre-chilled eppendorfs. 2µl β-mercaptoethanol was added to each aliquot with gentle mixing. Cells were incubated on ice for 10 min with gentle mixing every 2 min. 5µl *DpnI*-treated sample DNA was added to the cell aliquots and incubated on ice for 30 min. Tubes were heat-shocked in a 42°C water bath for precisely 30s and immediately transferred to ice for 2-3 min. 0.5ml chilled LB broth was added to each tube and incubated at 37°C for 1h in an orbital shaker (140rpm). In order to prepare LB/Agar plates for blue-white colour screening, 50µl 10mM IPTG (Promega) and 50µl 50mg/ml X-Gal (Promega) were spread over the surface and incubated face up at 37°C for 30 min prior to plating transformations. Sample reactions were centrifuged at 1000rpm for 10 min and the pellet resuspended in 200µl LB. The whole reaction was then spread on the prepared agar plates and left to air-dry for 10 min before incubating upside down overnight at 37°C.

2.2.6.3 Screening of Clones: Minipreps

Screening of transformants was carried out by small-scale preparation of plasmid DNA followed by appropriate restriction digests and/or sequencing. Single colonies from transformed plates were inoculated into 1.5ml LB/Ampicillin and incubated overnight at 37°C in a rotating oven (180rpm). Picked colonies were also patched

onto LB/Agar/Ampicillin plates and incubated overnight at 37°C. The following morning, cultures were poured into eppendorfs and spun for 1 min at 13000rpm. The supernatant was removed and the pellet resuspended in 100µl GTE. Lysis was achieved with the addition of 200µl 0.2M NaOH/1%SDS and vigorous vortexing. Cells were incubated on ice for 15 min before the addition of 150µl potassium acetate (pH 4.8) and vigorous vortexing. After a further 15 min incubation on ice, samples were centrifuged for 5 min at 13000rpm. 450µl of supernatant was removed and added to an equal volume of phenol/chloroform/IAA with vortexing. After a 2 min centrifugation at 13000rpm, the upper aqueous layer was removed and added to 2 vol 100% ethanol. Samples were incubated at room temperature for 5 min, centrifuged for 5 min at 13000rpm and allowed to air-dry. Pellets were resuspended in 50µl TE + 1µl RNase A (1mg/ml).

2.2.6.4 Large scale plasmid DNA preparation: CsCl density gradient centrifugation

Large scale plasmid DNA preparations were carried out on all novel plasmid DNA constructs and all plasmids used in transient transfection assays. Following transformation, single colonies were picked into 2ml LB/Ampicillin and incubated at 37°C for approximately 6h in an orbital shaker (180rpm). The cultures were then used to inoculate 500ml LB/Ampicillin and incubated at 37°C overnight in an orbital shaker (160rpm). Cells were collected by centrifugation in 250ml centrifuge pots at 6000rpm for 5 min at 4°C using a Beckman JA-14 rotor in a Beckman J2-MC centrifuge. The bacterial pellet was completely resuspended in 12ml GTE (per 500ml culture), followed immediately with the addition of 24ml freshly prepared 0.2M NaOH/1%SDS and vigorous shaking. After a 10 min incubation on ice, 16ml cold potassium acetate was added, shaken vigorously and the mix replaced on ice for a further 10 min. Bacterial debris was removed by centrifugation at 6000rpm for 5 min (as previously) and filtered through 2 layers of gauze into fresh 250ml centrifuge pots. DNA was precipitated by adding 32ml isopropanol and incubating for 30 min at room temperature followed by centrifugation at 10000rpm for 3 min. After removal of supernatant, pellets were allowed to air-dry for at least 1h. At this stage, pellets

were resuspended in 2.2ml TE before the addition of 2.95g CsCl (to give a final CsCl concentration of 1.67g/ml). 100µl ethidium bromide was added in order to visualise the DNA and the solution was carefully transferred into 3ml Beckman Quikseal tubes. Samples were centrifuged in a TLA100.3 rotor in a Beckman Optima TLX centrifuge at 20°C. After an initial spin at 70000rpm for 18h, banded plasmid DNA was withdrawn through the tube wall using a needle and syringe and transferred into a new ultracentrifuge tube. Tubes were filled with TE/CsCl (prepared by adding 100g CsCl to 100ml TE) and re-spun at 100000rpm for 4h. Banded DNA was withdrawn as described previously and ethidium bromide was removed by repeated isopropanol extractions until the pink colour had completely disappeared. Salt was removed by dialysis against 3 changes of TE for 24h at 4°C. DNA concentration was measured using a GeneQuant spectrophotometer (Pharmacia Biotech) before storage at –20°C.

2.2.7 Transient Transfection

2.2.7.1 Calcium phosphate precipitation method

Transient transfection by the calcium phosphate precipitation method was carried out in B103 cells as first described by Graham & van der Eb (Graham & van der Eb, 1973). All transfections were carried out in triplicate to at least n=5 using at least two independently prepared plasmid DNAs isolated by CsCl density gradient centrifugation. Promoter activity was determined by luciferase expression while transfection efficiency was controlled for by β-galactosidase activity. Cells were harvested by centrifugation in a Heraeus Labofuge 400R at 1200rpm for 3 min followed by resuspension in DMEM plus serum to give 2 x 10^5 cells/ml. 1ml of cells were added to 60mm dishes containing 3ml DMEM plus serum and incubated at 37°C with 5% CO_2 for 24h before transfection. Medium was replaced at least 1h prior to transfection and all transfection solutions were equilibrated to room temperature before use. Two tubes were prepared for each transfection, the first containing a total of 10µg DNA and 37µl 2M $CaCl_2$, made up to 300µl with dH_2O, and the second containing 300µl 2 x HBS. Control DNA solutions were as follows:

(i) 10μg pGEM3 (negative control), (ii) 5μg pSV2 luciferase (positive control) + 4μg pGEM3 + 1μg pCH110. In addition to reporter plasmid, each experimental DNA solution contained 1μg pCH110 (β-Galactosidase expression plasmid) and DNA concentration adjusted with pGEM3 to give a total of 10μg. For transfections involving autoregulation by GR, an expression plasmid encoding human GR, pRShGR was included at either 10 or 100ng. Each tube of 2 x HBS was gently vortexed while the DNA solution was added dropwise, and incubated for 30min at room temperature. Solutions were vortexed again before dropwise addition to the transfection plates with gentle agitation. Medium was refreshed 24h post-transfection. Dexamethasone was added, where appropriate, at least 1h post-transfection. 'Treated' cells were given 4.6μl 1mM or 10μM dexamethasone (in ethanol) to give final concentrations of 1 x 10^{-6}M and 1 x 10^{-8}M dexamethasone respectively. 'Untreated' cells were given 4.6μl ethanol. Treatments were repeated 1h after the medium was changed, 24h after transfection. 48h post-transfection, cells were washed carefully in 1 x PBS and incubated in 300μl lysis buffer for 20 min. Transfection dishes were scraped with rubber policemen and cell lysates were transferred into eppendorf tubes. Cell debris was pelleted by centrifugation at 13000rpm for 2 min and assays were carried out on the cell lysates immediately.

2.2.7.2 Cationic lipid method

Transient transfection using lipofectin (Invitrogen), a cationic lipid, was carried out in H4IIE cells. Cells were seeded at 2 x 10^5 cells in 4ml DMEM plus serum and incubated for 24h before transfection at 37°C with 5% CO_2. Two tubes were prepared for each transfection, the first containing 4μg DNA adjusted to 200μl with Opti-MEM (Invitrogen), the second containing 16μl lipofectin adjusted to 200μl with Opti-MEM. Control transfections contained (i) 4μg pGEM3 (ii) 2μg pSV2 luciferase + 1μg pGEM3 + 1μg pCH110. In addition to reporter plasmid, experimental DNA solutions contained 1μg pCH110 and pGEM3 adjusted to give a total DNA concentration of 4μg. Tubes were allowed to stand at room temperature for 30-45 min before being mixed together gently and incubated for a further 15 min. 1.6ml

Opti-MEM was then added to each tube containing the lipofectin-DNA complexes. Cells were washed with 3ml Opti-MEM before the transfection solution was gently overlaid. Cells were incubated for 6h at 37°C after which the transfection solution was replaced with 4ml DMEM plus serum. Cells were harvested 48h post-transfection as described above.

2.2.7.3 Luciferase Assays

Luciferase assays were carried out in duplicate on each triplicate transfection sample, with all solutions equilibrated to room temperature prior to use. 5µl 30mM ATP (Sigma), 100µl Luciferase Assay Buffer and 40µl cell lysate were combined in 5ml Röhren tubes (Sarstedt). Luciferase activity was assayed over 10s using a Lumat LB9501 luminometer which injected 105µl 1mM beetle luciferin (Promega) into each sample. The mean of each duplicate reading was recorded.

2.2.7.4 β-Galactosidase Assays

β-Galactosidase assays were carried out in duplicate using a Galacto-Light Plus kit (Tropix) equilibrated to room temperature before use. Fresh Galacton Plus reagent was diluted 1:100 in its Reaction Buffer Diluent and 67µl of the diluted solution was added to 10µl cell lysate. Samples were incubated at room temperature for 30-45min and β-Galactosidase activity was assayed in the luminometer as described above, which injected 105µl Light Emission Accelerator reagent. The mean of each duplicate reading was again recorded.

2.2.7.5 Transfection Data Analysis

The mean of each duplicate luciferase and β-galactosidase reading was entered into a Microsoft Excel spreadsheet for analysis. The negative pGEM3 control reactions provided background data for both luciferase and β-galactosidase assays; mean luciferase and β-galactosidase values pGEM3 were calculated and subtracted from

all experimental values. Promoter activity was expressed as luciferase activity/β-Galactosidase activity in order to control for variations in transfection efficiency between dishes. Values were expressed relative to the appropriate control vector (pGL3-BM or pGL3 Promoter) given an arbitrary value of 1, or, where appropriate, relative to P2 given an arbitrary value of 100%.

2.2.8 Protein techniques

2.2.8.1 Preparation of nuclear extracts for the analysis of protein-DNA interaction

Nuclear extracts from B103 or H4IIE cells were prepared as described by Dignum et al (Dignam et al., 1983). The cells from 20 x F75 flasks were dislodged either by tapping (B103) or treatment with trypsin (H4IIE) as described in section 2.2.1. Cells were pooled and collected by centrifugation in an Heraeus Labofuge 400R at 1200rpm for 5 min at 4°C. The pellet was resuspended in 5 x packed cell volumes (PCV) of 1 x PBS and centrifuged as before. Cells were resuspended in 5 x PCV of pre-chilled Nuclear Extract Buffer A (NEBA) and counted using a Neubauer Improved Haemocytometer. Following a 10 min incubation on ice, cells were spun as before and resuspended in 2 x PCV NEBA. The solution was transferred to a pre-chilled Dounce 1ml all-glass homogeniser and the cells lysed with 10 strokes of the tight pestle. Lysis was confirmed by microscopic analysis (x10 magnification) and the solution was spun as before. The supernatant was removed and the remaining pellet was transferred to a pre-chilled sterile 50ml Beckman tube. Further centrifugation was carried out using a Beckman JA-20 rotor in a Beckman J2-MC centrifuge at 10000rpm for 20 min at 4°C. A total of 3ml Nuclear Extract Buffer C (NEBC) per 10^9 cells was used to resuspend the pellet. The solution was then transferred to a pre-chilled Dounce 1ml all-glass homogeniser and lysed by 10 strokes of the tight pestle. The suspension was transferred to a pre-chilled glass beaker and stirred with a magnetic bead for 30 min at 4°C before centrifugation in a Beckman J2-MC centrifuge at 10000rpm for 30 min at 4°C. The supernatant was retained and dialysed against 50 x volumes of pre-chilled Nuclear Extract Buffer D

(NEBD) for 5h at 4°C. Following a final centrifugation in a Beckman J2-MC centrifuge at 10000rpm for 20 min at 4°C, the supernatant was aliquoted into pre-chilled eppendorf tubes and snap frozen in liquid nitrogen. Samples were stored at –80°C.

2.2.8.2 Estimation of protein concentration of extracts

Protein concentrations were estimated using the BioRad protein assay based on the Bradford method. The BioRad protein assay dye reagent concentrate was diluted 1:5 with dH_2O to give a working solution. A standard protein curve was constructed in duplicate by preparing standards containing 0, 1.5, 3, 6, 9, 12 and 15μg bovine βγ-globulin (made up to 10μl with dH_2O) in a standard 96-well plate. A range of sample concentrations were also prepared in duplicate (adjusted to 10μl with dH_2O), and 200μl BioRad reagent was added to both controls and samples before incubation at room temperature for 5-10 min. The plate was then inserted into a Bio-tek Instruments EL 312e microplate bio-kinetics reader and absorbance at 570nm was measured. A calibration curve was obtained from the standards, against which sample protein concentrations could be calculated.

2.2.9 Analysis of Protein-DNA interactions

2.2.9.1 Gel Mobility Shift Assays

Protein-DNA interactions were analysed by gel mobility shift assays as described by Singh et al (Singh et al., 1986). Specifically, reactions were carried out in a total volume of 30μl incorporating 6μl 5 x Gel Mobility Shift Assay Buffer, 3μg non-specific DNA competitor poly (dI-dC).(dI-dC), 0-40μg protein extract and either 30fmol [^{32}P]-labelled DNA fragment or 0.1pmol [^{32}P]-labelled double-stranded oligonucleotide. Reactions were incubated for 15 min at room temperature prior to the addition of [^{32}P]-labelled DNA and a further 15 min prior to loading onto a non-denaturing polyacrylamide gel (section 2.2.2.4).

Competition gel mobility shift assays were carried out using 10- or 100-fold molar excess of unlabelled specific or non-specific competitor fragments or oligonucleotides. Competitors were added to gel shift reactions just prior to the addition of [^{32}P]-labelled DNA. Gel Mobility Supershift Assays were also undertaken, in which antibodies to specific transcription factors were included in the reactions and further retarded protein-DNA complexes during electrophoresis. Specifically, 1µg antibody was included in the pre-incubation prior to the addition of [^{32}P]-labelled DNA.

2.2.9.2 *DNaseI* Protection Analysis

DNaseI 'footprinting' analysis of protein-DNA binding was undertaken as described by Galas & Schmitz (Galas & Schmitz, 1978). Reactions were prepared in a total volume of 40µl containing 8µl 5 x DNase I Protection Buffer, 1µg poly(dI-dC).(dI-dC) and 0-50µg protein extract. Following incubation on ice for 15 min, ~200000 cpm radiolabelled DNA (see section 2.2.4.8) was added. Reactions were further incubated on ice for at least 30 min. Samples were treated with 0.2-0.5U *DNaseI* for *exactly* 1 min and reactions were stopped with 10µl *DNase I* Footprinting Stop Solution. Samples were purified by phenol/chloroform extraction; 50µl phenol/chloroform/IAA was added to each sample and vortexed vigorously for 15s. After centrifugation at 12000g for 2 min, the upper aqueous layer was transferred to a fresh tube and extracted with 50µl chloroform. After further vortexing and centrifugation, the upper layer was again transferred to a fresh eppendorf tube. DNA samples were precipitated with 1µl tRNA (10µg/µl) and 125µl 100% ethanol and incubated on dry ice for no more than 7 min. DNA was collected by centrifugation at 12000g for 10 min at room temperature. Pellets were washed with ice-cold 100% ethanol and air-dried for 5-10 min before resuspension in 4-8µl formamide loading buffer (Promega). Samples were denatured for 10 min at 100°C before loading onto a denaturing polyacrylamide gel as described in section 2.2.2.5.

2.2.10 Analysis of Chromatin: *DNaseI* Hypersensitive Site Mapping

2.2.10.1 Preparation of Nuclei

7 x F75 flasks of confluent H4IIE cells were used to prepare nuclei for *DNaseI* hypersensitive site mapping. First, the medium was aspirated and the cells were washed twice in 1 x PBS. 3ml trypsin was applied to each flask followed by incubation at 37°C for 2 min. After dislodging the adherent cells, 7ml FCS was added to each flask and the mixture transferred to 2 x 50ml falcon tubes. Cells were collected by centrifugation in an Heraeus Labofuge 400R at 1200rpm for 3 min at 4°C and carefully resuspended in a total of 5ml pre-chilled Nuclei Buffer A (NBA). A small aliquot was removed at this stage for later comparison. In order to lyse the cell membrane, 5ml pre-chilled NBB was added and the mixture incubated on ice for 3 min. Nuclei were pelleted by centrifugation at 1200rpm for 3 min at 4°C and carefully resuspended in 5ml pre-chilled NBC. A further centrifugation at 1200rpm for 3 min at 4°C was followed by resuspension in 0.5ml NBC. The presence of intact nuclei at this stage was confirmed by comparison with the earlier, pre-lysis aliquot under 40 x magnification. A sample of nuclei was digested in 1% SDS and approximate DNA concentration was estimated by A_{260} measurement on a Shimadzu UV-160A spectrophotometer. After dilution with cold NBC to give a solution of 10 A_{260} units/ml, nuclei were kept on ice and used immediately.

2.2.10.2 *DNaseI* Treatment

100µl aliquots of nuclei (section 2.2.10.1) were treated with *DNaseI* at 30, 40 and 50 U/ml for exactly 0, 1, 2, 4 and 8 min. Enzyme activity was stopped with 100µl *DNaseI* Stop Solution. Each reaction was treated with 5µl RNaseA to remove RNA and incubated with proteinase K (at a final concentration of 100mg/ml) for 2h at 37°C. Samples were purified as described in section 2.2.4.3 and subject to Southern analysis as described in 2.2.4.3.

3 Analysis of GR alternate exon 1 expression in a variety of rodent cell lines.

3.1 Introduction & Aims

Evidence discussed in Chapter 1 generated the hypothesis that the multiple exon 1 structure of the GR promoter may be involved in the tissue-specific regulation of GR levels. This hypothesis implies that tissues show specific patterns of GR exon 1 expression.

Previous work carried out in this laboratory identified eleven potential alternate exons 1 (termed 1_1, 1_2, 1_3, 1_4, 1_5, 1_6, 1_7, 1_8,1_9,1_{10} and 1_{11}) (McCormick et al., 2000), five of which have also been described in mouse (Strahle et al., 1992; Chen et al., 1999) and three in human (Breslin et al., 2001; Nunez & Vedeckis, 2002) (see fig.1.3). Further analysis demonstrated that expression of exons 1_5 and 1_7 was hippocampus-specific, exon 1_1 expression was thymus-specific and expression of exons 1_6 and 1_{10} was ubiquitous (McCormick et al., 2000; McCormick, 2000). Correspondingly, studies of human and mouse tissues showed that exons 1B and 1C (homologous to rat exons 1_6 and 1_{10} respectively) were also ubiquitously expressed, albeit at varying levels (Strahle et al., 1992; Breslin et al., 2001; Nunez & Vedeckis, 2002).

Further to the *in vivo* data described above, the experiments detailed in this chapter aimed to identify rodent cell lines in which alternate GR exons 1 are differentially expressed. In this way, cell lines displaying specific patterns of GR exon 1 expression could be compared and manipulated with relative ease. Cell lines have the additional advantage of cell type homogeneity, compared to the often heterogeneous nature of tissues. A variety of rodent cell lines representing a range of tissues were therefore screened for GR alternate exon 1 expression. In identifying cell lines that could satisfactorily model *in vivo* tissues, it was also important to ensure that absolute levels of GR were comparable. As such, a quantitative analysis of GR mRNA in various rodent cell lines was also undertaken.

3.2 Experimental Design

3.2.1 RT-PCR analysis of GR alternate exon 1 expression in rodent cell lines.

To investigate the cell specific expression of GR alternate exons 1, non-quantitative RT-PCR analysis was performed on a range of rodent cell lines including H4IIE (rat hepatoma), KNRK (rat kidney), PC12 (rat pheochromocytoma), NG-108 (rat glioma), EL-4 (mouse lymphoma) and S-49 (mouse lymphoma). First strand cDNA synthesis was carried out using an oligo(dT) primer and PCR reactions were performed using 5' primers specific for individual GR exons 1 in conjunction with a common 3' primer complementary to GR exon 2 (section 2.1.7.1). Positive control reactions for each primer pair were carried out on rat thymus (exon 1_1) and rat liver (exons 1_4, 1_5, 1_6, 1_7, 1_{10} and 1_{11}). In order to control for DNA contamination of RNA samples, negative control reactions were also carried out for each cell sample and primer pair, in which the reverse transcription step was carried out in the absence of AMV Reverse Transcriptase enzyme. Where appropriate, RT-PCR reactions were carried out in order to detect 'total' GR using 5' and 3' primers specific for exons 2 and 3 respectively. Predicted PCR product sizes are given in table 3.1.

Transcript	Predicted size (bp)
1_1	345
1_4	436
1_5	353
1_6	353
1_7	347
1_{10}	422
1_{11}	363
GR	630
GAPDH	1001

Table 3.1 *Predicted sizes of PCR products.*

3.2.2 De-methylating treatment of a cell line lacking GR expression.

The hypothesis that methylation of the GR locus is responsible for the absence of GR expression in the B103 cell line was tested by treating cells with the de-methylating

agent 5-aza-2'-deoxycytidine (5-Aza-CdR). If methylation has 'silenced' the GR locus, treatment with 5-Aza-CdR should restore GR expression. Cells were therefore grown in 60mm dishes for 24h in the presence or absence of 2μM 5-Aza-CdR. Following extraction, RNA sample integrity was first verified by denaturing gel electrophoresis. Subsequently, RT-PCR analysis was carried out to detect GR mRNA, with the inclusion of appropriate positive and negative controls (as described in section 3.2.1).

3.2.3 Quantitative RT-PCR analysis of GR expression in rodent cell lines.

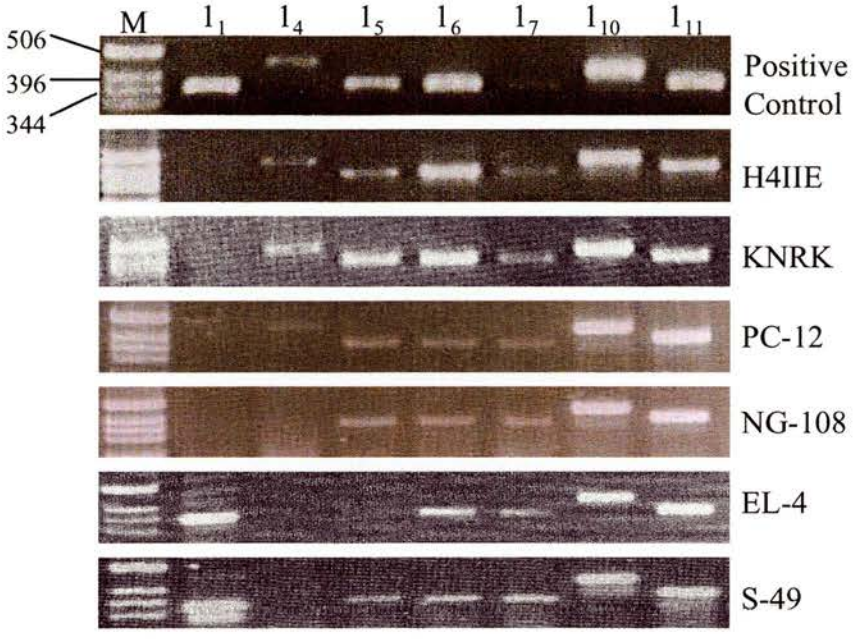
Quantitative RT-PCR was carried out in order to establish model rodent cell lines that express GR at levels equivalent to *in vivo* tissues. The rat GR competitor used in these experiments has been described previously (Lai et al., 2003; section 2.1.8). The quantitative RT-PCR strategy is based on the principle that the GR competitor yields a truncated product (550bp) in relation to endogenous GR (630bp) when PCR using GR-specific primers is performed. As such, reactions were carried out using a series of known competitor concentrations combined with a constant amount of sample RNA. At the point when the two PCR products show equal intensity, the concentration of endogenous mRNA is equivalent to that of exogenously added competitor (see section 2.2.5.3.1 for details of calculation method).

3.3 Results

3.3.1 All alternate exons 1 located in the CpG island region are present in all rodent cell lines tested, but exon 1_1 expression is restricted to T cells.

GR exon 1-specific RT-PCR reactions were carried out on H4IIE (rat hepatoma), KNRK (rat kidney), PC-12 (rat pheochromocytoma), NG-108 (rat glioma), EL-4 (mouse lymphoma) and S-49 (mouse lymphoma) cells. Results show that products corresponding to the predicted sizes of all alternate exons 1 examined were expressed in all cell lines tested (fig. 3.1). The exception was the exon 1_1 product, which was restricted to the T cell lines EL-4 and S-49. It should be noted that several

Figure 3.1 *'CpG island' exons 1 are present in all rodent cell lines tested whilst exon 1_1 expression is restricted to cells of the immune system.*
Representative RT-PCR using primers specific to individual GR exons 1 in conjunction with a common GR exon 2 primer. RT reactions were performed using 4μg RNA extracted from the following cell lines: H4IIE (rat hepatoma), KNRK (rat kidney), PC-12 (rat adrenal pheochromoctyoma), NG-108 (rat glioma), EL-4 (mouse lymphoma) and S-49 (mouse lymphoma), which were subsequently divided between exon 1-specific PCR reactions. Positive control reactions were carried out on rat thymus (exon 1_1) and rat liver (exons 1_1, 1_4, 1_5, 1_6, 1_7, 1_{10} and 1_{11}). Negative control reactions were also carried out for each primer pair in each cell line using RT reactions minus AMV reverse transcriptase. No bands were observed in the negative controls (data not shown). 1kb ladder was run in lane M and the 344, 396 and 506bp bands are indicated.


M
1
4
5
6
7
10
11
506
396
344
Positive Control
H4IIE
KNRK
PC-12
NG-108
EL-4
S-49

PCR products, particularly 1_1, appeared as a doublet. Sequence analysis showed that these products differed in the size of a $(CAG)_n$ repeat located at the beginning of exon 2 (J.Nixon, personal communication).

3.3.2 GR is not expressed in B103 cells

No products from individual GR exon 1 transcripts were observed when the RT-PCR analysis described above was carried out on B103 (rat neuroblastoma) cells (data not shown). Further RT-PCR analysis was carried out to determine if B103 cells indeed express GR. The integrity of B103 RNA was first verified by denaturing agarose gel electrophoresis (data not shown). Subsequently, RT-PCR reactions using primers specific to both GR and the positive control gene GAPDH were performed. GAPDH mRNA was present in both rat liver and B103 cells, while GR mRNA was only detected in liver (fig. 3.2). No significant bands were observed in the negative control reactions. B103 cells do not, therefore, appear to express GR.

3.3.3 5-aza-2'-deoxycytidine treatment does not restore GR expression in B103 cells.

To test the hypothesis that methylation of the GR CpG island region has 'silenced' GR expression, B103 cells were treated with the known de-methylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR). RT-PCR reactions were then performed on RNA extracted from rat liver as well as 5-Aza-CdR treated and untreated B103 cells, using primers specific for GR and the positive control gene GAPDH.

GAPDH mRNA was shown to be present in both untreated and 5-Aza-CdR treated B103 cells (fig. 3.3). However, GR mRNA was not detected in untreated B103 cells, nor was it restored following treatment with 2μM 5-Aza-CdR. The integrity of the GR reaction was confirmed by the detection of a GR product in rat liver. Surprisingly, in this experiment no GAPDH transcript was detected in rat liver RNA, although it was clearly present in other experiments (e.g. figure 3.2). The reason for the lack of GAPDH product observed in this experiment is unknown. No bands were

Figure 3.2 *GR is not expressed in B103 cells.*
RT-PCR using primers specific for GR and GAPDH carried out on 4μg RNA extracted from rat liver ('L') or B103 ('B') cells. Reactions were performed in the presence (+) or absence (-) of reverse transcriptase (RT). 1kb ladder was run in lane M and the 1636bp, 1018bp and 506bp markers are labelled. Products corresponding to GAPDH (1001bp) and GR (630bp) are also indicated.

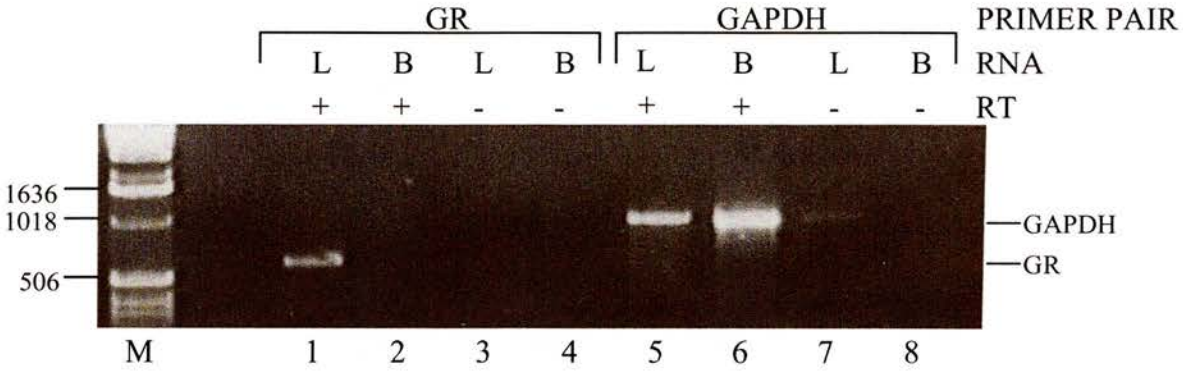
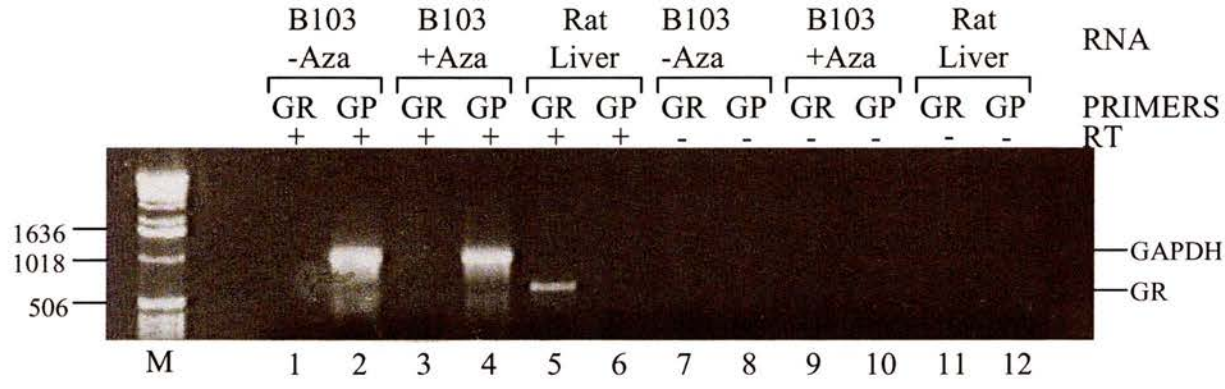

GR
GAPDH
PRIMER PAIR
L B L B L B L B
RNA
+ + - - + + - -
RT
1636
1018
506
GAPDH
GR
M 1 2 3 4 5 6 7 8

Figure 3.3 *5-aza-2'-deoxycytidine treatment does not restore GR mRNA expression in B103 cells.*
RT-PCR using primers specific for GR or GADPH (GP) on 4μg RNA extracted from either rat liver or B103 cells grown in the presence or absence of 2μM 5-aza-2'-deoxycytidine (Aza). Reactions were performed in the presence (+) or absence (-) of reverse transcriptase (RT). 1kb ladder was run in lane M and the 1636bp, 1018bp and 506bp markers are labelled. Products corresponding to GAPDH (1001bp) and GR (630bp) cDNAs are also indicated.


B103
-Aza
B103
+Aza
Rat
Liver
B103
-Aza
B103
+Aza
Rat
Liver
RNA
GR GP GR GP GR GP GR GP GR GP GR GP
PRIMERS
+ + + + + + - - - - - - RT
1636
1018
506
GAPDH
GR
M 1 2 3 4 5 6 7 8 9 10 11 12

observed in any of the negative control reactions. It appears, therefore, that treatment with the de-methylating agent 5-Aza-CdR does not restore GR expression in B103 cells.

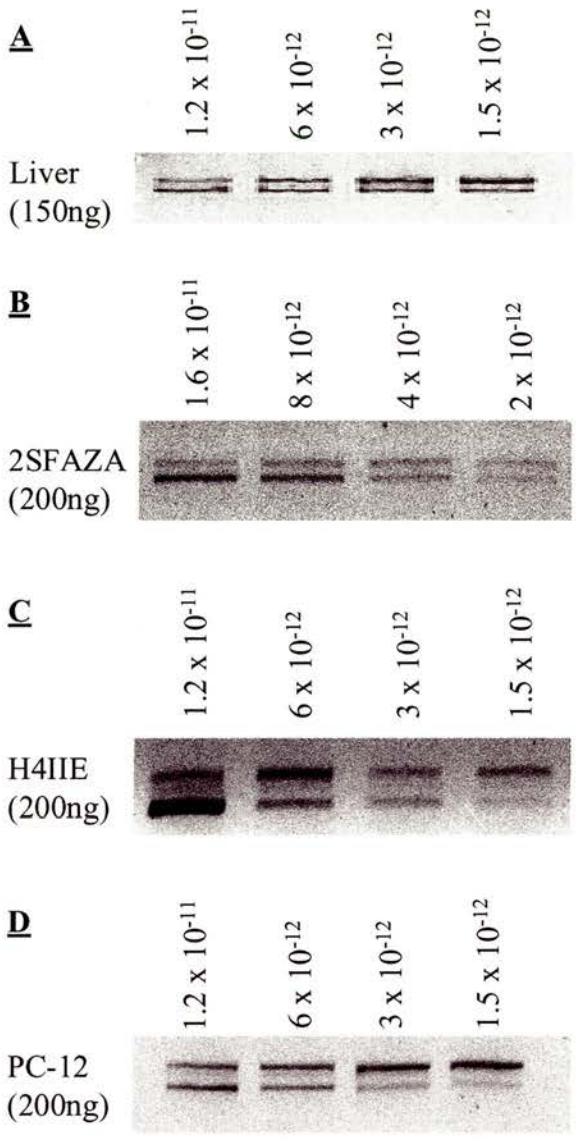
3.3.4 Competitive RT-PCR analysis of GR expression in H4IIE, 2S FAZA and PC-12 cells.

Measurement of absolute GR mRNA levels in rat liver as well as H4IIE (rat hepatoma), 2S FAZA (rat hepatoma) and PC-12 (rat pheochromocytoma) cells was carried out using competitive RT-PCR. Representative RT-PCR results showed the presence of both 630bp and 550bp products, produced from endogenous and competitor GR RNA respectively, in all samples (fig. 3.4A, rat liver; B, 2S FAZA; C, H4IIE; D, PC-12). H4IIE cells were found to express GR mRNA at a concentration of 2.4×10^7 copies/μg total RNA, very similar to the levels of 2.5×10^7 copies/μg total RNA detected in rat liver (fig. 3.4E). In addition, 2S FAZA and PC-12 cells were shown to express GR levels of 8.5×10^5 and 1.7×10^6 copies/μg total RNA respectively.

3.4 Discussion

Experiments described in this chapter aimed to identify rodent cell lines that could satisfactorily model GR expression observed *in vivo* tissues. Specifically, overall levels of GR mRNA and patterns of alternate GR exon 1 expression were investigated. Results described here showed that in all cell lines tested, including H4IIE (rat hepatoma), KNRK (rat kidney), PC-12 (rat pheochromocytoma), NG-108 (rat glioma), EL-4 (mouse lymphoma) and S-49 (mouse lymphoma) cells, products corresponding to GR exons 1_4, 1_5, 1_6, 1_7, 1_{10} and 1_{11} were present. It should be noted that variability in detection of the 1_4 product may be due to its general low abundance. Previous work has shown that exon 1_4-containing transcripts are relatively rare (McCormick et al., 2000; Freeman, 2003). Consistent with this, the exon 1_4 transcript could be detected in all cell lines under RT-PCR conditions in

Figure 3.4 *Competitive RT-PCR measurement of GR mRNA levels in rat liver, 2S FAZA, H4IIE and PC-12 cells.*
Representative gels showing competitive RT-PCR analysis using GR-specific primers on 150ng or 200ng RNA extracted from rat liver (A), 2S FAZA (B), H4IIE (C) and PC-12 (D) cells. RT reactions were supplemented with a synthetic GR competitor at levels between 1.6×10^{-11} and 1.5×10^{-12} moles as shown. Products of 630bp and 550bp, generated by endogenous and competitor RNA respectively, are labelled. Panel E shows the absolute levels of endogenous GR mRNA for each cell type. Calculations were performed as described in section 2.2.5.3.1.



E

Cell Type	Moles/ug total RNA	No. copies/ng total RNA
Rat Liver	4.2×10^{-17}	2.5×10^{7}
2SFAZA	1.4×10^{-18}	8.5×10^{5}
H4IIE	4.0×10^{-17}	2.4×10^{7}
PC-12	2.8×10^{-18}	1.7×10^{6}

which more RNA template was included or the number of amplification cycles was increased (data not shown).

It was interesting that a consistent relationship between the intensities of alternate GR exon 1 products was observed in most samples. Products corresponding to exons 1_{10}, 1_{11} and 1_6 showed the greatest intensities in all cell lines tested, which might be expected from the higher levels of exons 1_6 and 1_{10} previously observed in rat tissues (McCormick et al., 2000; McCormick, 2000). While it must be remembered that PCR is a non-quantitative technique, these data suggest that absolute levels of alternate exons 1 may vary between tissues. Nevertheless, all alternate GR exons 1 located in the CpG island region were expressed to some degree in all cell lines tested. In theory, RT-PCR can detect a single mRNA transcript present in a sample and is therefore a more sensitive technique than RPA. As such, while transcripts corresponding to the 'minor' exons 1_4, 1_5, 1_7 and 1_{11} were below the limit of detection of the RPA analysis carried out by McCormick *et al*, (McCormick et al., 2000), they were readily identified by the more sensitive RT-PCR technique used in these experiments. Indeed, further RT-PCR analysis carried out in this laboratory has identified all 'CpG island' alternate exons 1 in all rat tissues tested (Freeman, 2003).

These results are consistent with data gained from mouse cells and tissues showing that expression of the 'CpG island' exons 1B, 1C, 1D and 1E (homologous to rat exons 1_6, 1_{10}, 1_5 and 1_{11} respectively) is ubiquitous (Strahle et al., 1992; Chen et al., 1999). Similarly, human GR exons 1B and 1C (equivalent to 1_6 and 1_{10}) have been detected in all human cell lines investigated, including Jurkat (T-cell ALL), HeLa (cervical carcinoma), HepG2 (hepatoma), IM-9 (B-cell lymphoma), CEM-C7 (T-cell lymphoblasts), HL-60 (myeloid leukaemia), MCF-7 (breast carcinoma), 786-0 (kidney carcinoma), SJSA (osteosarcoma), H1299 (lung carcinoma) and WI-38 (fibroblasts) (Breslin et al., 2001; Nunez & Vedeckis, 2002). Work carried out in this laboratory has also identified human homologues to the rat GR exons 1_4, 1_5, 1_7, 1_8, 1_9 and 1_{11} (Reynolds, 2002). It would be interesting to determine if, as the current evidence suggests, these additional exon 1 variants are ubiquitously expressed in human cells and tissues.

It appears therefore that cells do not operate an 'all-or-nothing' policy with regards to expression of the GR 'CpG island' exons. Rather, transcription is initiated at all CpG island exons 1 in all cells. Regulation of GR does not occur as a result of switching the transcription of alternate exons 1 'on' or 'off'. If the GR CpG island exons do indeed play a role in the regulation of GR, it must be through adjustment of their relative levels.

Exon 1_1-containing mRNA is not expressed in H4IIE, KNRK, PC-12 or NG-108 cells but is present in the lymphoma cell lines EL-4 and S-49. In contrast to the exon 1_4 reaction, the 1_1 product was repeatedly only detected in lymphoma cells, despite alterations to RT-PCR parameters. In accordance with previous work, it appears that rat exon 1_1 expression is restricted to cells of the immune system (McCormick et al., 2000; Freeman 2003). In contrast to the CpG island alternate exons 1, it may be the case that expression of exon 1_1, which is located ~30.2kb upstream of exon 2, is subject to regulation by a tissue-specific promoter.

Studies carried out in human cells have shown that alternative splicing events generate three different exon 1A (homologous to rat exon 1_1)-containing transcripts, 1A1, 1A2 and 1A3 (Breslin et al., 2001). It should be noted that, despite presumably sharing a common promoter, the expression of only one variant, 1A3, was restricted to cells of the immune system. In contrast, exons 1A1 and 1A2 were both detected in cell lines representing a variety of human tissues (Breslin et al., 2001). Similarly, RPAs demonstrated that exon 1A mRNA was present at low levels in a range of mouse tissues (Chen et al., 1999). Work is currently being undertaken in this laboratory to investigate if rat tissues also express the three exon 1_1 variants and, as such, should establish if the exon 1_1 region is associated with a tissue-specific promoter.

While glucocorticoid exposure is usually associated with down-regulation of GR expression, one report showed that exon 1A1-, 1A2- and 1A3- containing transcripts were up-regulated in CEM-C7 (human ALL) cells following hormone treatment

(Breslin et al., 2001). The same report suggested that expression of exon 1A facilitates up-regulation of GR in T cells, causing them to undergo programmed cell death or apoptosis. It is interesting to note that cells such as EL-4 and S-49, shown in these experiments to express exon 1_1, also expressed all other alternate exons 1 examined. This corresponds to data reported for human T cells and mouse thymus, in which exons 1B, 1C, and (in mouse), 1D and 1E (homologous to rat exons 1_5 and 1_{11} respectively) were also detected (Chen et al., 1999; Breslin et al., 2001). Speculation into the functional consequences of exon 1_1 expression must therefore consider that exon 1_1-containing transcripts form only a proportion of total GR mRNA. Indeed, previous RPAs showed that transcripts containing exon 1_1 were present at ~25% in rat thymus, although the proportion of exon 1_1 expression in specific thymus cell subtypes was not addressed (McCormick, 2000).

In light of previous work showing the ubiquitous expression of GR (Ballard et al., 1974) it was surprising to identify a cell line, B103 (rat neuroblastoma), in which GR was absent. There are several possible causes for the lack of GR expression in B103 cells. One hypothesis tested in this study was based on work carried out by Antequera *et al* which showed that in cell lines, *de novo* methylation can occur at the CpG islands of genes that are non-essential in culture (Antequera et al., 1990). Many studies have shown an inverse correlation between transcriptional activity of a gene and the presence of CpG methylation (reviewed in Bird, 1984). In order to investigate if methylation was indeed 'silencing' GR expression in the B103 cell line, cells were treated with the de-methylating agent, 5-aza-2'-deoxycytidine (5-Aza-CdR). This treatment has previously been shown to restore the expression of several genes in cultured cells (Harris, 1984). Results described here show that 5-Aza-CdR treatment did not restore GR expression in B103 cells. Thus, methylation of the GR locus does not appear to be responsible for the lack of GR expression in this cell line. However, it should be noted that the efficacy of 5-Aza-CdR treatment was not controlled for in this experiment. It cannot, therefore, be ruled out that the lack of GR mRNA expression in treated B103 cells is due to the 5-Aza-CdR not achieving the desired de-methylation effect.

An alternative possibility for the lack of GR mRNA expression in B103 cells is a mutation, most probably a deletion, at the GR locus. This could be tested by Southern or PCR analysis of B103 genomic DNA. Whatever the cause behind the lack of GR mRNA expression, it is interesting to note that cells in which this 'housekeeping' gene is absent are clearly viable. COS-1 (green monkey kidney) cells and the parallel cell line CV-1 have previously been shown to lack detectable GR protein, although the cause of the absence has not been identified (Giguere et al., 1986). Similarly, HepG2 (human hepatoma) cells have been shown to express functional GR but at a level too low to stimulate glucocorticoid-responsive gene transcription (Baumann, et al., 1990). Given the enormous number of GR-responsive genes and their involvement in normal development and physiological homeostasis, it is surprising that cells in which functional GR expression is either minimal or absent are perfectly viable. With regard to the B103 cell line, it effectively forms a neural GR 'knock-out' and could therefore be used to investigate any cell-specific downstream implications of a lack of GR expression.

It should also be remembered that glucocorticoids tend to be anti-proliferative in most, but not all, tissues (Crocker et al., 1998; Sakai et al., 1999; Almawi & Tamim, 2001). It is therefore conceivable that a lack of GR expression might be advantageous in cultured cells, where frequent passaging selects for rapid growth and proliferation. It was previously assumed that the housekeeping roles of GR required its expression in virtually every cell. However, these results suggest that cell lines in which GR has been lost may be more common than expected.

Finally, quantitative analysis of GR mRNA levels in rat liver as well as 2S FAZA (rat hepatoma), H4IIE (rat hepatoma) and PC-12 (rat pheochromocytoma) cells showed H4IIE cells to express GR at levels most similar to rat liver. Furthermore, the levels of GR mRNA detected in rat liver and H4IIE cells during these experiments (2.5×10^4 and 2.4×10^4 copies/ng total RNA respectively) were comparable with the value of $18.65 \pm 6.85 \times 10^3$ copies/ng total RNA previously reported for rat liver (Zhang & Byrne, 2000). Interestingly, these experiments showed that the 2S FAZA hepatoma cell line expressed GR at levels ~30-fold less than both rat liver and

H4IIEs. 2S FAZAs are therefore another example of a cell line in which GR levels are considerably lower than expected, perhaps again suggesting that culture conditions might select against high GR expression.

The aim of these experiments was to identify a model cell line in which the observed GR levels were comparable to those found *in vivo*, and, in this respect, the H4IIE result is encouraging. However, it should be noted that competitive RT-PCR results for all samples were subject to considerable variability and the results presented here should be viewed with caution. Representative results are shown in figure 3.4 but several more reactions, in which the results did not reach sufficient statistical power, were carried out for each cell line. The relative rarity of the GR transcript is reflected in the small quantities of both endogenous and competitor mRNA involved in these reactions. Such low template RNA levels may have contributed to the observed variability in this study and further experiments to increase 'n' are required.

To conclude, these experiments demonstrated that exon 1_1 was the only GR alternate exon 1 to show tissue-specific expression, being restricted to cells of the immune system. In contrast, all exons 1 located in the CpG island region were expressed in all rodent cell lines tested. In addition, the rat neuroblastoma cell line, B103, was shown to be viable in the total absence of GR mRNA expression. However, methylation of the GR locus did not appear to be responsible for the lack of GR expression in these cells. Finally, the absolute level of GR mRNA detected in H4IIE (rat hepatoma) cells was shown to be comparable with that observed in rat liver. This result, together with RT-PCR data showing the presence of all alternate exons 1 examined, suggested that H4IIE cells are a good model for rat liver and, as such, they were selected for use in further experiments.

4 Investigation of promoter elements involved in rat GR regulation.

4.1 Introduction & Aims

Results described in the previous chapter showed that all alternate GR exons 1 of the CpG island region were detected in all rodent cell lines examined, indicating that cells do not operate an 'all-or-nothing policy' with regard to expression of these exons 1. However, the alternate exon 1 structure of the GR CpG island region may still play a role in tissue-specific regulation; it may be the case that alternate exons 1 are driven by multiple promoters showing differential activity between cell types.

However, while the 3' ends of all identified exons 1 have been precisely mapped, most of the exact 5' transcription starts remain undefined. The GR promoter lacks any notable TATA or CAAT boxes, or even a consensus Initiator sequence (Zong et al., 1990; Encio et al., 1991; Gearing et al., 1993). Consistent with this, 5'RACE-PCR and primer extension analyses of rat and human GR have shown that the transcription starts of most alternate exons 1 are heterogeneous (Zong et al., 1990; Gearing et al., 1993; McCormick et al., 2000). All these factors have increased the difficulty in identifying exon 1–associated promoters.

Previous work has established that the CpG island region of GR exhibits significant promoter activity in human, rat and mouse cells (Strahle et al., 1992; Nobukuni et al., 1995; McCormick et al., 2000; Breslin et al., 2001; Nunez & Vedeckis, 2002). Several of these studies explored the possibility that alternate exons 1 are associated with individual, tissue-specific promoters. Work carried out in this laboratory demonstrated that promoter activity associated with rat exon 1_7 is neural-specific (McCormick et al., 2000). In addition, RPA analyses of selected mouse tissues and cells have shown variability in the absolute levels of exons 1B and 1C (Strahle et al., 1992). A series of reporter constructs in which the human GR promoter was fused within exon 1C, identified a region shown to bind the transcription factor AP2, which, when deleted, caused a cell-specific decline in promoter activity (Nobukuni et al., 1995). Nunez & Vedeckis also showed that luciferase reporter constructs

containing sequences proximal to either human GR exons 1B or 1C exhibited differential promoter activity between Jurkat (T-cell ALL) and HepG2 (hepatoma) cells, but not HeLa cells (cervical carcinoma) (Nunez & Vedeckis, 2002). Furthermore, Breslin *et al* showed no difference in the activity of promoter 1B between IM-9 (B lymphoma), CEMC-7 (T-cell lymphoblast), Jurkat, HeLa cells, with higher activity in WI-38 (fibroblast) cells than IM-9 cells but not significantly different to the other cell lines (Breslin et al., 2001). However, there were significant difference in the activity of promoter 1C between all the cell lines (Breslin et al., 2001).

The work described in this chapter was designed to (i) explore the hypothesis that alternate rat GR exons 1 are driven by individual promoters showing tissue-specific activity and (ii) further elucidate elements of the CpG island region involved in GR regulation. A series of constructs, in which various fragments of the rat GR CpG island region were fused to a luciferase reporter gene, were transiently transfected into two rat cell lines, B103 (neuroblastoma) and H4IIE (hepatoma). These cell lines were chosen based on previous work showing that B103 cells exhibit a neural-specific pattern of GR exon 1 promoter activity (McCormick et al., 2000), while results described in chapter 3 showed that H4IIE cells provide a physiologically relevant model for GR expression in rat liver.

4.2 Experimental Design

4.2.1 Transient Transfections

In order to investigate promoter activity over the GR CpG island region, a variety of constructs, in which elements of the GR promoter were fused to a luciferase reporter gene, were transiently transfected into B103 and H4IIE cells. The 3' GR deletion series used in these experiments, in which the 3' ends of individual rat GR exons 1 were fused directly to a luciferase reporter gene, has been described previously (McCormick et al., 2000; McCormick, 2000). While all members of the series share the same 5' end, the luciferase fusion in each construct abolishes the splice acceptor

site normally present at the beginning of exon 2. As such, only the fused exon contributes to luciferase activity; all upstream transcripts are spliced at their respective donor sites, but in the absence of a suitable acceptor site, are unlikely to give rise to functional luciferase activity. The 3' deletion series can therefore be seen to measure only the promoter activity associated with each individual exon 1. In contrast, the exon 2 splice acceptor site is retained in the 'P2' construct, which therefore reflects the activity of the whole promoter region.

A 5' deletion series of the GR promoter, constructed by V.Lyons, was also used in transient transfection experiments. Constructs in this series retain the same 3' end fused to luciferase, and were therefore used to assess the combined activity of multiple exons 1. In addition, a 5' deletion series of the $P1_7$ construct was used to localise a 134bp *PstI* fragment associated with neural-specific promoter activity and has been described previously (McCormick, 2000). To investigate any enhancer properties associated with the region, a further construct, in which the orientation of the 134bp *PstI* fragment was reversed ($P1_{7bREV}$), was also synthesised.

To investigate the contribution to overall promoter activity made by the 134bp *PstI* fragment, a series of constructs carrying internal deletions within P2 was synthesised by V. Lyons. Finally, potential promoter and enhancer properties of the 134bp *PstI* fragment were further investigated using plasmids constructed by V. Lyons in which the fragment was cloned in both orientations into a promoterless vector (pGL3-BM) and a vector containing a heterologous promoter (pGL3-Promoter).

4.2.2 *DNaseI* footprinting analysis of the exon 1_7 region.

In order to identify *trans*-acting proteins that may bind to the exon 1_7 region, *DNaseI* nuclease protection assays were undertaken. This assay works on the simple principle that a sequence-specific DNA binding protein protects the nucleotides involved in DNA binding from enzymatic digestion by *DNaseI*, leaving a 'footprint' (Galas & Schmidt, 1978). Experiments used two ^{32}P probes, F_A and F_B, which were generated from the 134bp *PstI* fragment (fig.2.1). Each probe was uniquely labelled

at one end and on one strand only. A further ^{32}P-labelled probe, F_C, corresponding to the 274bp *PstI/BglII* fragment located immediately downstream of the 134bp *PstI* region, was also used (see fig.2.1).

4.2.3 Electrophoretic Mobility Shift Assays of the 134bp *PstI* region

Electrophoretic Mobility Shift Assays (EMSAs) were used to further investigate the nature of protein-DNA interactions in the 134bp *PstI* region. This assay separates radioactively labelled 'free' DNA from protein-DNA complexes by non-denaturing polyacrylamide gel electrophoresis. The appearance of a DNA fragment with altered mobility (a 'shift') is therefore indicative of protein binding. The inclusion of an antibody specific to the bound protein can cause further retardation of the complex during gel electrophoresis, known as a 'supershift'. In addition to the labelled fragments described in 4.2.2, double stranded oligonucleotides representing both known transcription factor binding sites as well as specific segments of the 134bp *PstI* fragment were also used. Competition EMSAs, in which the ability of unlabelled fragments or oligonucleotides to compete with the labelled probe for binding to protein extracts, were also carried out to determine the specificity of interactions in protein-DNA complexes.

4.3 Results

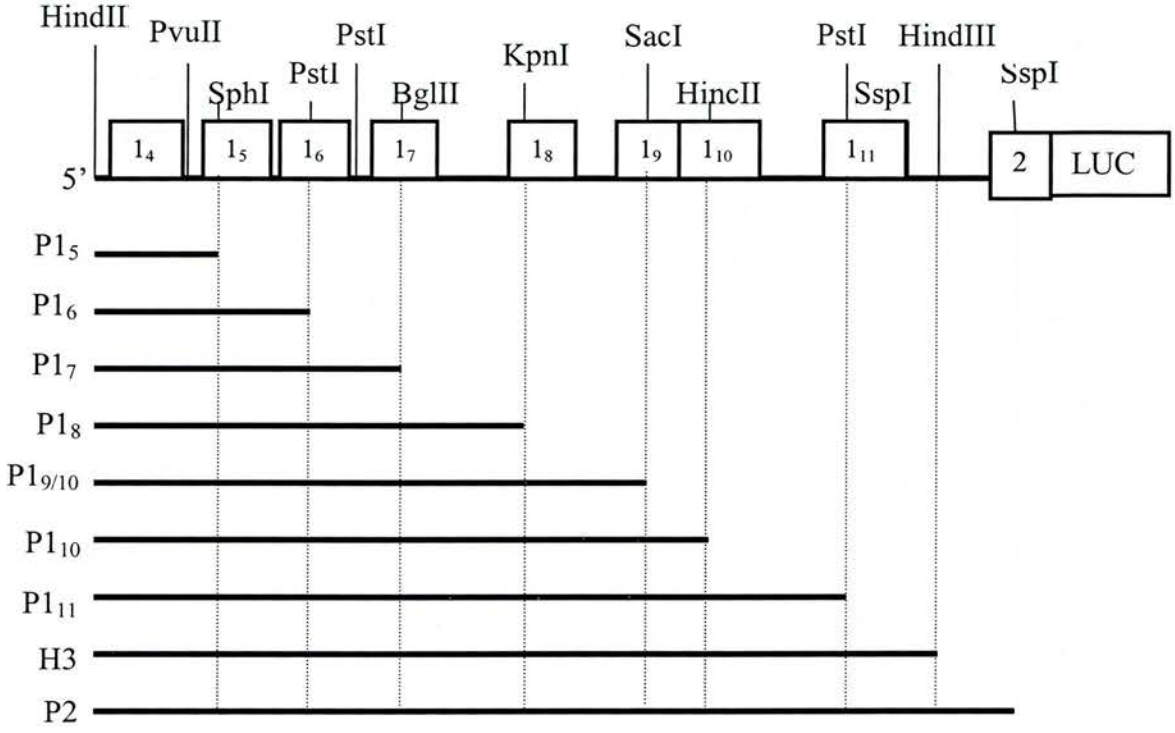
4.3.1 Promoter elements of the rat GR CpG island

4.3.1.1 High neural cell specific promoter activity is associated with $P1_7$.

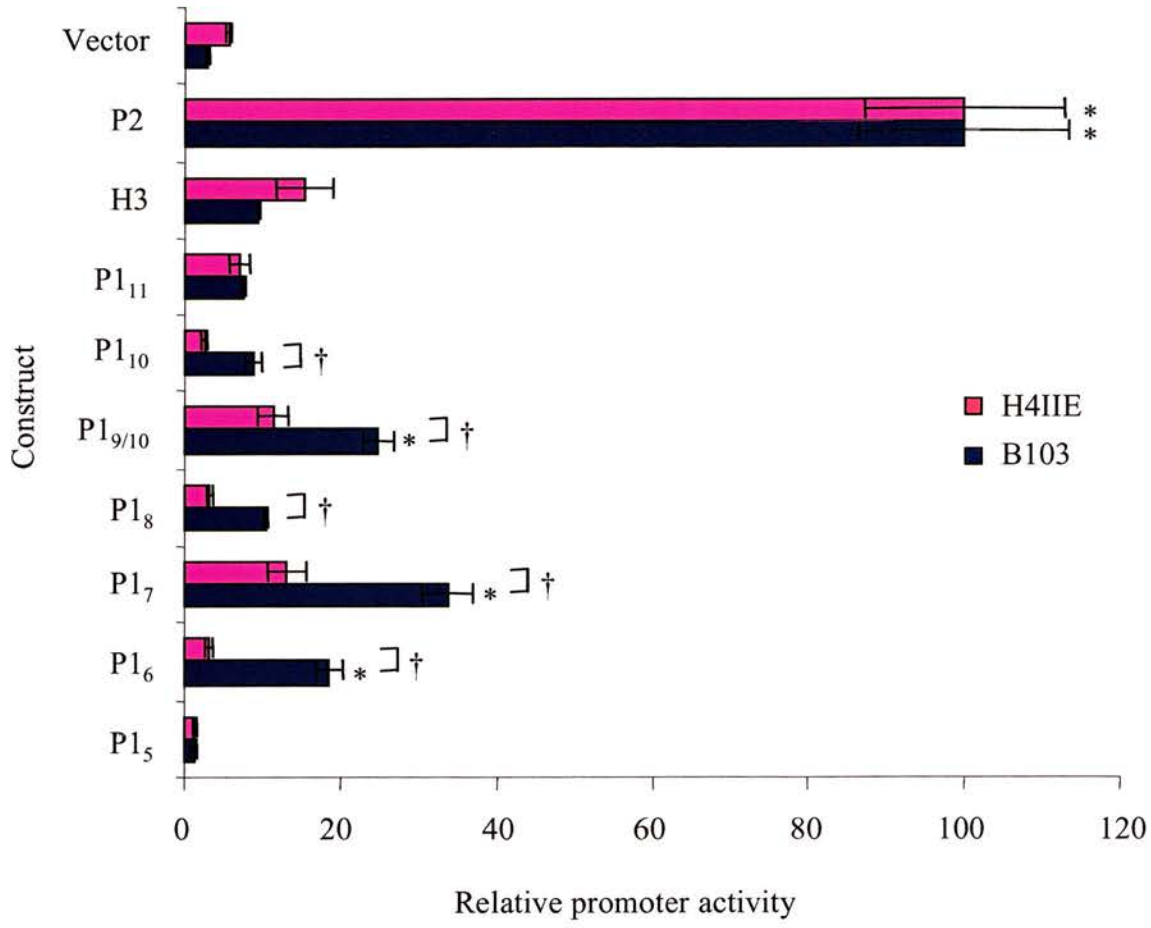
A 3' deletion series of the rat GR CpG island region was transfected into two rat cell lines, B103 (neuroblastoma) and H4IIE (hepatoma), in order to investigate cell-specific promoter activity associated with alternate exons 1 (fig. 4.1). The P2 construct, which represents the whole promoter, showed the highest activity in both

Figure 4.1 *High neural cell specific promoter activity is associated with $P1_7$.*
Transient transfection analysis of a 3' deletion series of the rat GR promoter.
A: Diagram showing the design of the 3' deletion series, in which each construct is fused to a luciferase reporter gene. All constructs have the same 5' end but differ at the 3' end. The 3' end of P2 is fused to luciferase within exon 2 while all other constructs are fused within known exons 1. Restriction enzyme sites used to engineer the construct series are also indicated.
B: Transient transfections carried out in H4IIE (rat hepatoma) and B103 (rat neuroblastoma) cells. The activity of all constructs is expressed relative to P2 nominally set at 100%. n=3-12 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to two way ANOVA analysis before *post-hoc* comparison using Student's t-test. '*' denotes a significant difference from empty vector in the same cell line ($p<0.05$).). '†' denotes a significant difference from the same construct in the other cell line ($p<0.05$).
C: Transient transfection data compiled from H4IIE and B103 transfections shown in fig 4.1B with previous results for HepG2 (human hepatoma), C6 (rat glioma) and B103 (rat neuroblastoma) cells (McCormick et al., 2000). The activity of all constructs is expressed relative to a P2 value nominally set at 100%. n=3-12 (2 independent plasmid DNA preparations); values represent means ±SEM. Data were subject to two way ANOVA analysis before *post-hoc* comparison using Fisher's LSD test. '*' denotes a significant difference from both empty vector and the same construct in hepatoma cell lines ($p<0.05$). '†' denotes a significant difference from empty vector but not the same construct in other cell lines ($p<0.01$).

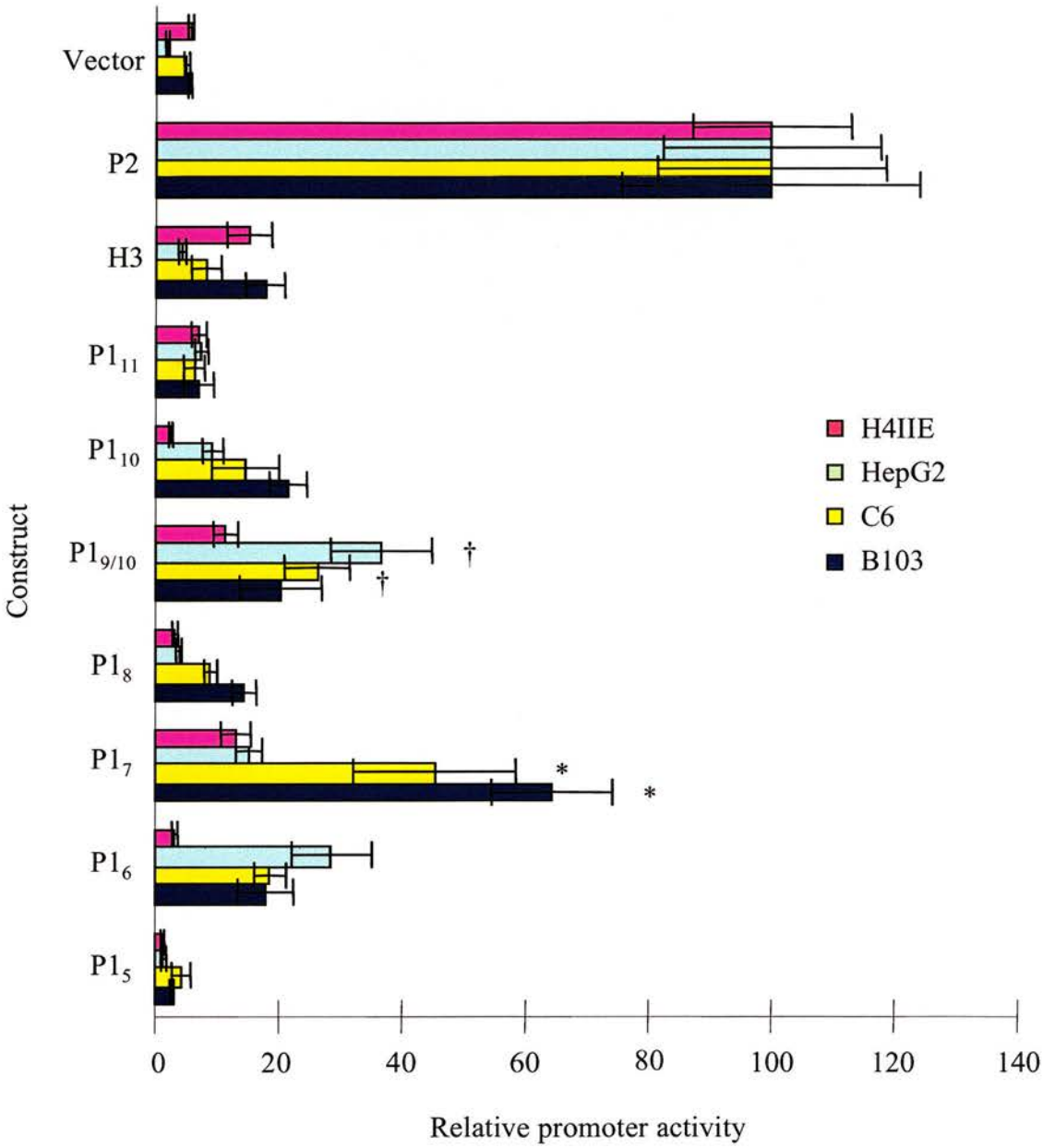
A


HindII
PvuII
SphI
PstI
PstI
BglII
KpnI
SacI
HincII
PstI
SspI
HindIII
SspI
1₄
1₅
1₆
1₇
1₈
1₉
1₁₀
1₁₁
2
LUC
5'
P1₅
P1₆
P1₇
P1₈
P1₉/₁₀
P1₁₀
P1₁₁
H3
P2

B


Vector
P2
H3
P1 11
P1 10
P1 9/10
P1 8
P1 7
P1 6
P1 5
Construct
H4IIE
B103
0
20
40
60
80
100
120
Relative promoter activity

C


Vector
P2
H3
P1 11
P1 10
P1 9/10
P1 8
P1 7
P1 6
P1 5
Construct
H4IIE
HepG2
C6
B103
†
†
*
*
0
20
40
60
80
100
120
140
Relative promoter activity

cell lines ($p<0.001$) (fig. 4.1B). No construct, apart from P2, showed significant promoter activity in H4IIE cells. In contrast, $P1_6$, $P1_7$ and $P1_{10}$ all showed significantly higher activity than empty vector in B103 cells ($p<0.05$).

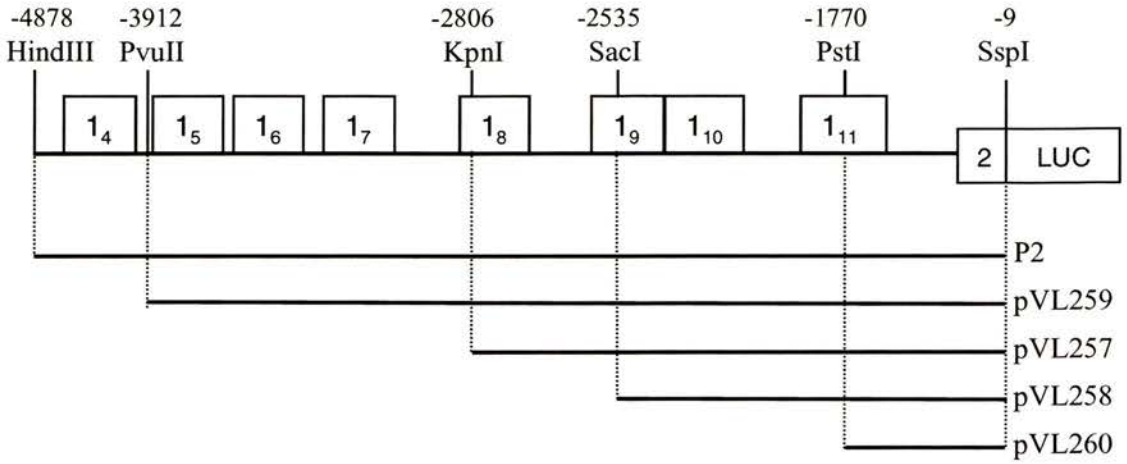
However, data gained for B103 cells, while confirming the pattern of exon 1-associated promoter activity previously observed (McCormick et al., 2000; McCormick 2000), were the result of only one transient transfection experiment (carried out in triplicate). As such, results from the current study were compiled with previous data obtained in this laboratory (McCormick et al., 2000; McCormick, 2000) (fig. 4.1C). The pattern of activity of the constructs was broadly similar in all cell lines. Again P2 shows significantly higher activity than all other constructs in all cell lines ($p<0.01$). In B103 cells, $P1_7$ shows significantly increased activity relative to all other constructs ($p<0.001$). In C6 cells $P1_{9/10}$ and $P1_7$ are significantly higher than empty vector ($p<0.05$), with $P1_7$ significantly higher than all other exon 1 constructs apart from $P1_{9/10}$ ($p<0.01$). Finally, in HepG2 cells, $P1_{9/10}$ is significantly higher than empty vector ($p<0.01$), and all other exon 1 constructs apart from $P1_6$ and $P1_7$ ($p<0.05$). The activity of $P1_7$ is significantly higher in the neural cell lines B103 and C6 than in the hepatoma cell lines H4IIE and HepG2 ($p<0.01$). The high promoter activity associated with $P1_7$ appears therefore to be neural specific. No other construct showed cell-type specific promoter activity.

4.3.1.2 Two regions of the GR promoter account for the majority of activity in B103 cells.

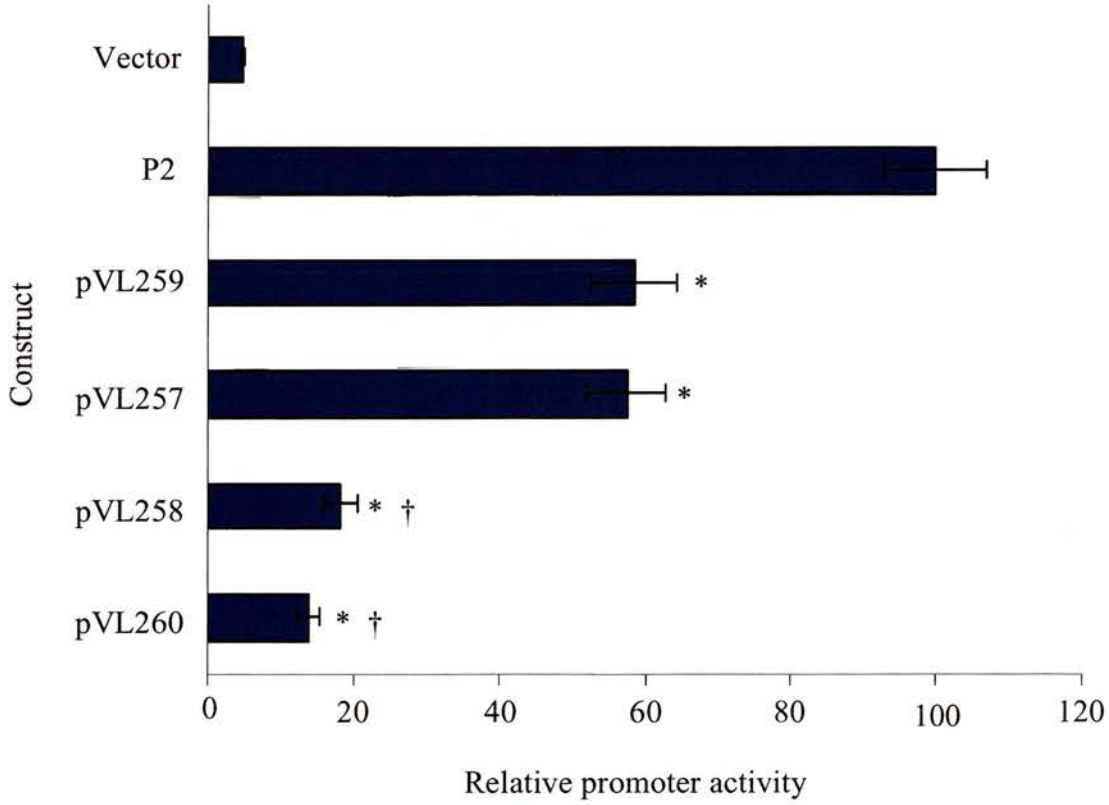
While the 3' deletion series of the GR promoter tested activity associated with individual exons 1, a 5' deletion series was used to investigate the combined activity of multiple exons 1 (fig. 4.2). All constructs in the series showed significant activity relative to empty vector in B103 cells ($p<0.001$) (fig. 4.2B). Results show that while a deletion from –4878 to –3912 (pVL259) resulted in a significant decrease in promoter activity of ~40% relative to P2 ($p<0.001$), a further deletion to –2806 (pVL257) caused no additional decrease in activity. Furthermore, while removal of a fragment from –4878 to –2535 (pVL258) caused a decrease in promoter activity of

Figure 4.2 *Two regions of the GR promoter account for the majority of activity in B103 cells.*
Transient transfections of a 5' deletion series of the GR promoter in B103 (rat neuroblastoma) cells.
A: Diagram showing the design of the 5' deletion series in which each construct is fused to a luciferase reporter gene. All constructs have the same 3' end but differ at the 5' end. The 3' end of each construct is fused to luciferase within exon 2. Restriction enzymes sites used to engineer constructs are also shown.
B: Transient transfections carried out in B103 cells. The activity of all constructs is expressed relative to P2 nominally set at 100%. n=16-21 (2 independent plasmid DNA preparations); values represent means ±SEM. Results were subject to analysis by Student t-test. '*' denotes a significant decrease from P2 ($p<0.001$). '†' denotes a significant decrease from pVL257 ($p<0.001$).

A


-4878
HindIII
-3912
PvuII
-2806
KpnI
-2535
SacI
-1770
PstI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P2
pVL259
pVL257
pVL258
pVL260

B


Vector
P2
pVL259
pVL257
pVL258
pVL260
Construct
*
*
* †
* †
0
20
40
60
80
100
120
Relative promoter activity

~80% relative to P2, an additional deletion to –1770 produced no further reduction. As such, two regions, the first between –4878 and –3912 (R_1) and the second between –2806 and –2535 (R_2), each account for ~40% of P2 promoter activity in B103 cells.

4.3.1.3 The 134bp region responsible for neural cell specific $P1_7$ promoter activity is orientation dependent.

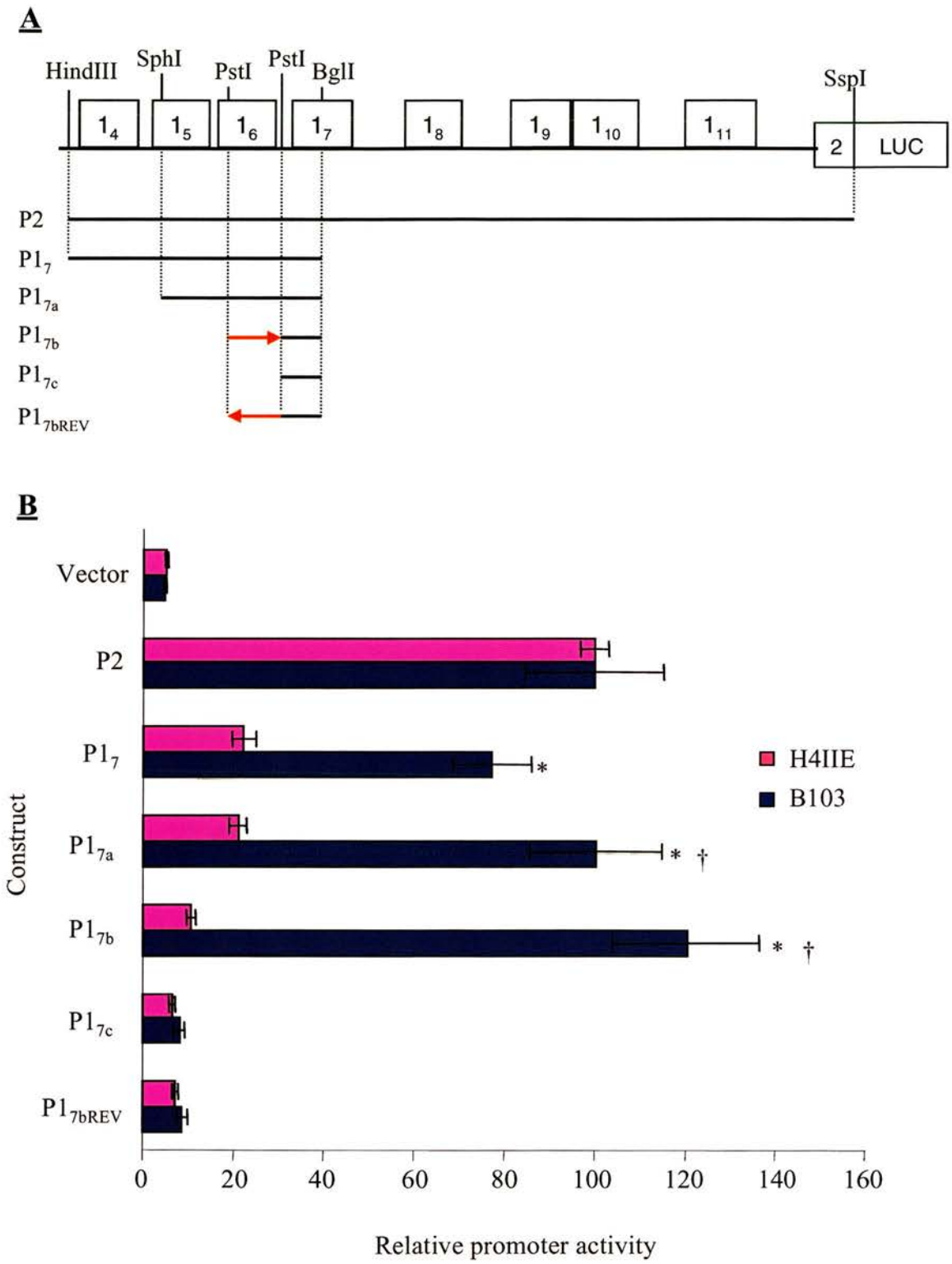
Previous work (McCormick, 2000), together with data presented in section 4.3.1.1, showed an association between the $P1_7$ construct and neural-specific promoter activity. Further investigations were carried out using a 5' deletion series of $P1_7$ transiently transfected into B103 and H4IIE cells (fig. 4.3). No exon 1_7-associated construct showed significantly higher activity than empty vector in H4IIE cells (fig 4.3B). Once again, in B103 cells $P1_7$ activity was significantly higher than that of empty vector ($p<0.001$) and $P1_7$ in H4IIE cells ($p<0.001$). $P1_{7a}$, in which a 881bp fragment is deleted from the 5' end of $P1_7$, showed a ~30% increase in promoter activity relative to $P1_7$ in B103 cells ($p<0.05$). Similarly, $P1_{7b}$, in which a further 216bp fragment is deleted from the 5' end of $P1_7$, showed an increase of ~56% in promoter activity relative to $P1_7$ ($p<0.01$). Importantly, deletion of an additional 134bp fragment from $P1_{7c}$ caused a ~90% decrease in promoter activity relative to $P1_7$ ($p<0.001$). It appears, therefore, that this 134bp *PstI* fragment is necessary for the high neural cell specific promoter activity associated with $P1_7$.

Potential enhancer properties of the 134bp *PstI* fragment were investigated using a construct based on $P1_{7b}$ in which the region was reversed ($P1_{7bREV}$). Interestingly, the activity of $P1_{7bREV}$ was identical to that of $P1_{7c}$, demonstrating that the 134bp *PstI* fragment is inactive in the reverse orientation (fig 4.3B).

4.3.1.4 The 134bp *PstI* fragment does not act as a classical enhancer

In order to confirm results presented in 4.3.1.3, in which the 134bp *PstI* fragment failed to show the orientation-independent activity associated with a traditional

Figure 4.3 *The 134bp region responsible for neural-specific $P1_7$ promoter activity is orientation dependent.*
Transient transfection analysis of the 5' deletion series of $P1_7$ in B103 (rat neuroblastoma) and H4IIE (rat hepatoma) cells.
A: Diagram showing design of the $P1_7$ 5' deletion series. All constructs, apart from P2, share the same 3' end, but differ at the 5' end. The 3' end of P2 is fused to luciferase within exon 2 while all other constructs are fused within exon 1_7. $P1_{7bREV}$ is a modified form of $P1_{7b}$ in which the 134bp *PstI* fragment is reversed. Restriction enzyme sites used to engineer the constructs are also shown.
B: Transient transfections carried out in B103 and H4IIE cells. The activity of all constructs is expressed relative to P2, nominally set at 100%. n=11-18 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to two way ANOVA analysis before *post-hoc* comparison using Fisher's LSD test. '*' denotes significant difference in construct activity between cell lines ($p<0.001$). '†' denotes significant increase in activity from $P1_7$ ($p<0.05$).


A
HindIII
SphI
PstI
PstI
BglI
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P2
P1 7
P1 7a
P1 7b
P1 7c
P1 7bREV
B
Vector
P2
P1 7
P1 7a
P1 7b
P1 7c
P1 7bREV
Construct
H4IIE
B103
*
* †
* †
0
20
40
60
80
100
120
140
160
Relative promoter activity

enhancer, the activity of the fragment was tested in a heterologous promoter vector, pGL3-Promoter (Promega). The 134bp *PstI* fragment failed to show significant activity in either the forward (pVL287) or reverse (pVL288) orientation in either B103 or H4IIE cells, confirming that it does not act as an enhancer in either cell line (fig 4.4).

4.3.1.5 The 134bp *PstI* fragment acts as an orientation dependent promoter.

Promoter properties associated with the 134bp *PstI* fragment were investigated using constructs in which the fragment was cloned in both orientations into a modified form of the promoterless vector pGL3-Basic (pGL3-BM). The fragment showed significant promoter activity in the forward orientation (pVL289) in both B103 and H4IIE cells ($p<0.001$) (fig. 4.5). Furthermore, the activity of the fragment was over 2-fold greater in B103 than in H4IIE cells ($p<0.001$). In contrast, no significant activity was observed in the reverse orientation in either cell line. Indeed, reversal of the 134bp *PstI* fragment caused a significant decrease in activity relative to empty vector in H4IIE cells ($p<0.001$). The 134bp *PstI* fragment therefore only confers promoter activity in the forward orientation, and is significantly more active in B103 than H4IIE cells.

4.3.1.6 Deletion of the 134bp *PstI* fragment from P2 causes a cell-specific decrease in activity.

A series of internal deletion constructs was used to investigate the contribution of the 134bp *PstI* fragment to the overall promoter activity of P2 (fig. 4.6). Deletion of the 134bp *PstI* fragment from P2 (pVL261) caused a significant decrease in promoter activity in B103, but not H4IIE cells ($p<0.01$) (fig. 4.6B). Deletion of a –3339 to –1770 region, which included the 134bp *PstI* fragment, from P2 (pVL262) reduced activity to levels not significantly different from empty vector in both cell lines. Interestingly, a deletion of –3205 to –1770 from P2 (pVL263), in which the 134bp *PstI* fragment is restored, also failed to show significant promoter activity in either

Figure 4.4 *The 134bp PstI fragment does not act as an enhancer.*
Transient transfections of constructs in which the 134bp *PstI* fragment was cloned in both the forward (pVL287) and reverse (pVL288) orientations into the heterologous promoter vector, pGL3-Promoter. Experiments were carried out in H4IIE (rat hepatoma) and B103 (rat neuroblastoma) cells. The activity of all constructs is expressed relative to empty vector nominally set at 1. n=6-12 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to two way ANOVA analysis before *post-hoc* comparison using Fisher's LSD test.

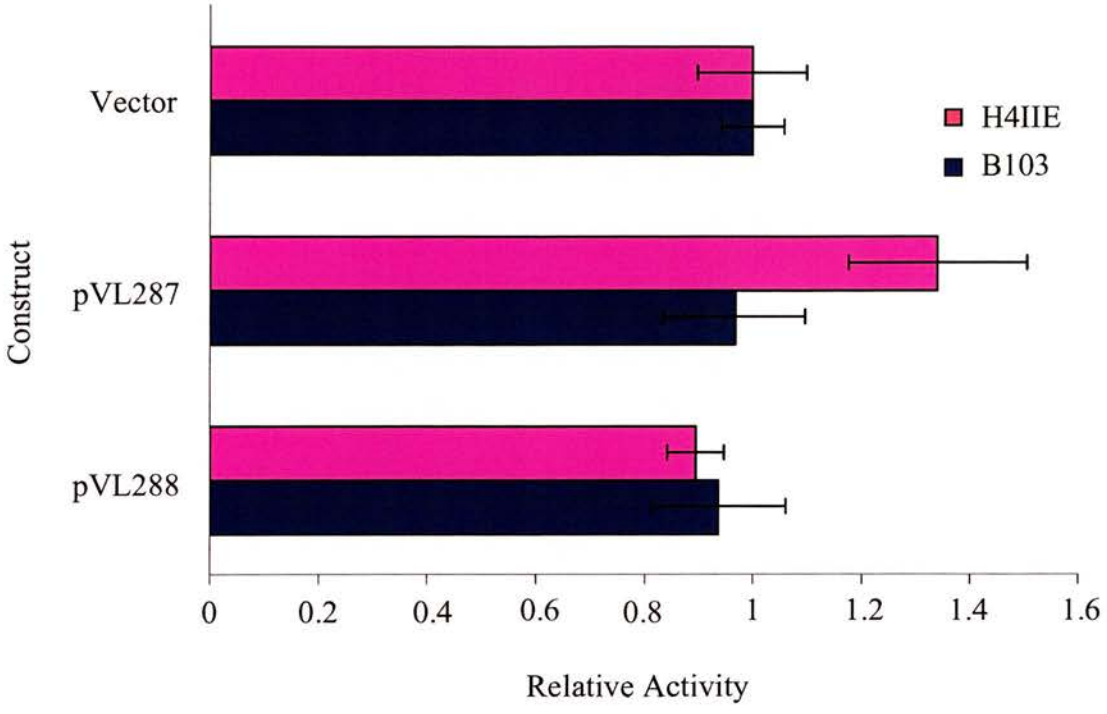

Vector
pVL287
pVL288
Construct
H4IIE
B103
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Relative Activity

Figure 4.5 *The 134bp PstI fragment acts as an orientation dependent promoter.* Transient transfections of constructs in which the 134bp *PstI* fragment was cloned in the forward (pVL289) and reverse (pVL290) orientations into pGL-3BM. Experiments were carried out in H4IIE (rat hepatoma) and B103 (rat neuroblastoma) cells. The activity of all constructs is expressed relative to empty vector nominally set at 1. n=13-18 (2 independent plasmid DNA preparations), means ±SEM. Values were subject to two way ANOVA analysis before *post-hoc* comparison using Fisher's LSD test. '*' denotes a significant difference from empty vector ($p<0.01$). '†' denotes a significant difference in construct activity between cell lines ($p<0.01$).

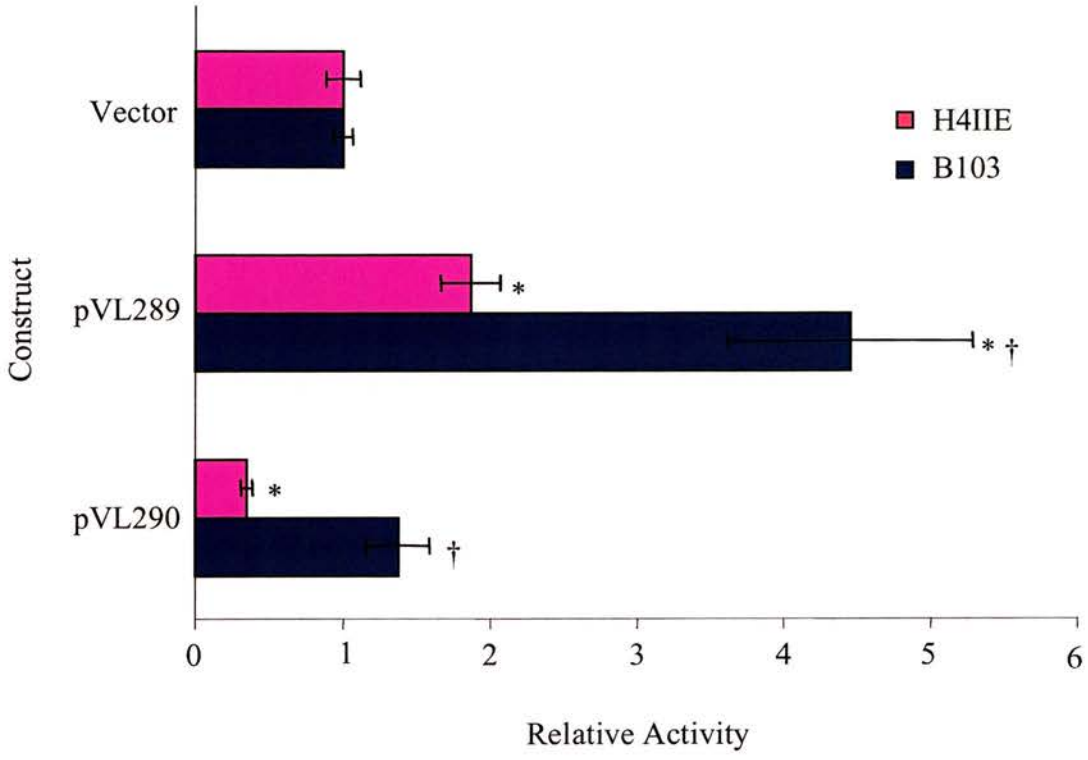
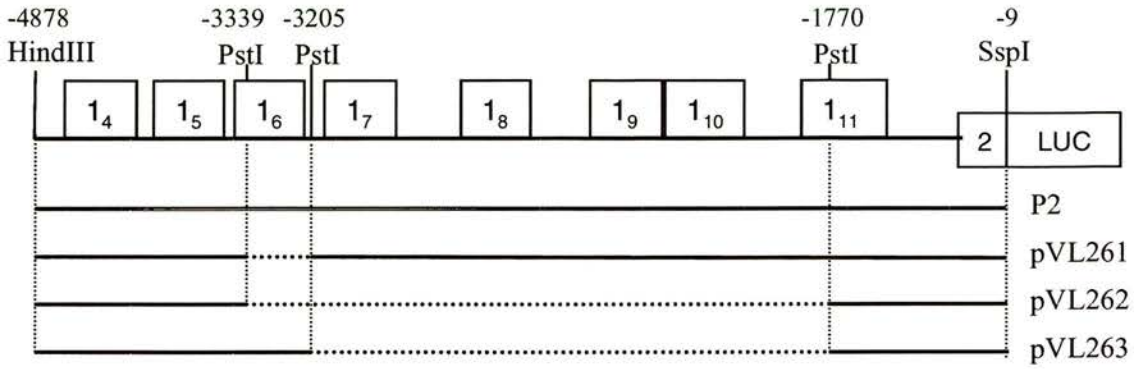
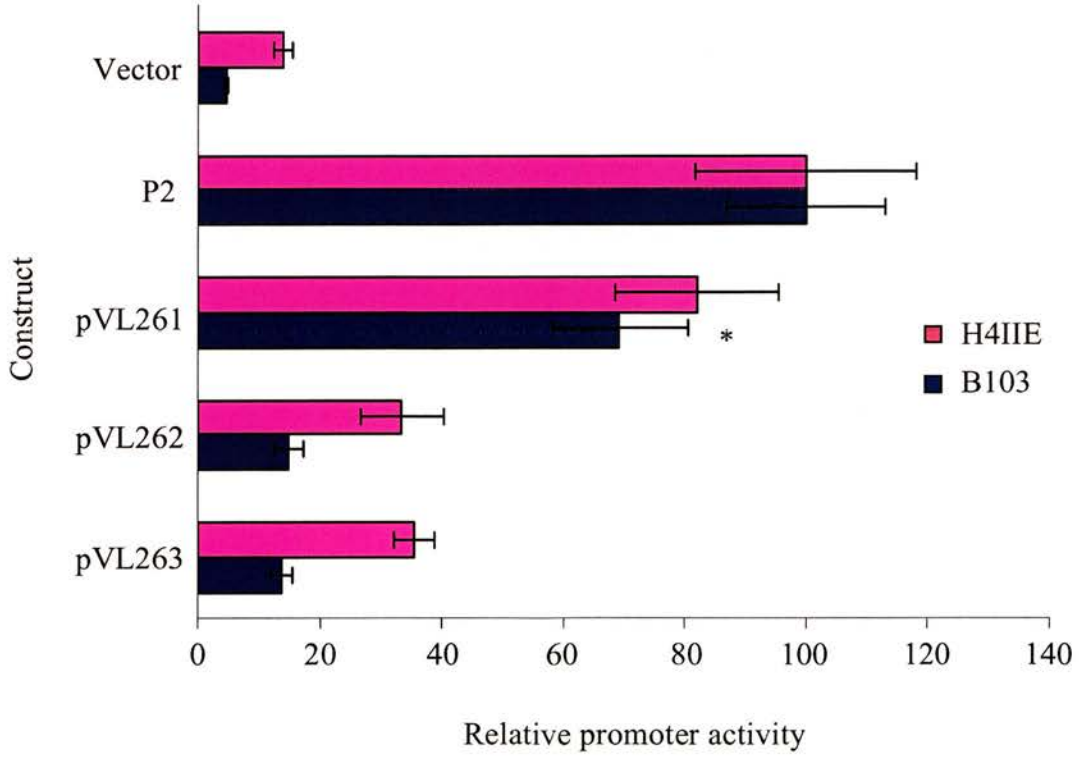

Vector
pVL289
pVL290
Construct
H4IIE
B103
*
* †
*
†
0
1
2
3
4
5
6
Relative Activity

Figure 4.6 *Deletion of the 134bp PstI fragment from P2 causes a cell-specific decrease in promoter activity.*
Transient transfections of *PstI* internal deletion constructs in H4IIE (rat hepatoma) and B103 (rat neuroblastoma) cells. A: Diagram showing the design of the *PstI* internal deletion constructs. All constructs have the same 5' and 3' ends but carry different internal *PstI* fragment deletions. The activity of all constructs was expressed relative to P2 nominally set at 100%. n=12-26 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to two way ANOVA analysis before *post-hoc* comparison using Fisher's LSD test. '*' denotes a significant difference from the activity of both empty vector and P2 ($p<0.01$).

A


-4878
HindIII
-3339
PstI
-3205
PstI
-1770
PstI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P2
pVL261
pVL262
pVL263

B


Vector
P2
pVL261
pVL262
pVL263
Construct
*
H4IIE
B103
0
20
40
60
80
100
120
140
Relative promoter activity

cell line. Therefore, following a deletion of –3205 to –1770 from the GR promoter, the presence of the 134bp *PstI* fragment does not 'rescue' promoter activity in either cell line.

4.3.1.7 There is no difference in activity between GR promoter constructs representing either –4878 to –3339, -1770 to -9 or both.

Comparison of constructs $P1_6$ (fig. 4.1C), pVL260 (fig. 4.2B) and pVL262 (fig. 4.6B) allows investigation of the contribution of two regions, -4878 to –3339 and –1770 to -9, to overall promoter activity (fig. 4.7). $P1_6$ possesses –4878 to –3339 only, pVL260 possesses –1770 to -9 only and pVL262 possesses both. Interestingly, there is no significant difference between the activities of any of the constructs.

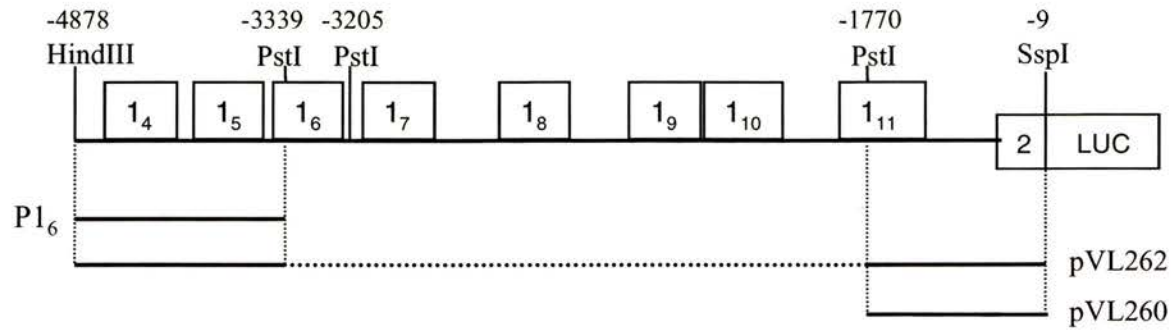
4.3.2 Protein-DNA interactions involving the 134bp *PstI* fragment

4.3.2.1 B103 and H4IIE nuclear extracts protect a 'footprint' on the 134bp *PstI* fragment from *DNaseI* digestion.

Sites and proteins involved in the neural-specific promoter activity of the 134bp *PstI* fragment were initially investigated by *DNaseI* footprinting. Experiments using the radiolabelled F_B fragment, which encompasses the 134bp *PstI* region, identified a footprint (FP1) at the 3' end of the fragment, produced by both B103 and H4IIE nuclear extracts (fig. 4.8). Due to the location of this footprint at the 3' end of the fragment, it was difficult to delineate its 3' boundary using the corresponding M+G sequencing reaction. However, the protected sequence consists of at least ten nucleotides between –3230 and –3220 and most probably extends for a further five or six nucleotides in the 3' direction. Comparison of this region with the TRANSFAC database suggested that the footprint might contain a putative AP2 transcription factor binding site (fig 4.8C).

Figure 4.7 *There is no difference in activity between GR promoter constructs representing either –4878 to –3339, -1770 to -9 or both.*
Compiled results of transient transfections of 3 constructs of the GR promoter in B103 (rat neuroblastoma) cells taken from data presented in figs 4.2, 4.3 and 4.7.
A: Diagram showing the design of the constructs. pVL262 (-4878 to –3330 plus –1761 to 0) and pVL260 (-1770 to 0) share the same 3' ends which are fused to luciferase within exon 2. In contrast, the 3' end of $P1_6$ (-4878 to –3330) is fused to luciferase within exon 1_6. Restriction enzymes sites used to engineer constructs are also shown.
B: Transient transfections carried out in B103 cells. The activity of all constructs is expressed relative to P2 nominally set at 100% (not shown). n=16-21 (2 independent plasmid DNA preparations); values represent means ±SEM. Data were subject to analysis by Student t-test.

A


-4878
HindIII
-3339
PstI
-3205
PstI
-1770
PstI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P1 6
pVL262
pVL260

B

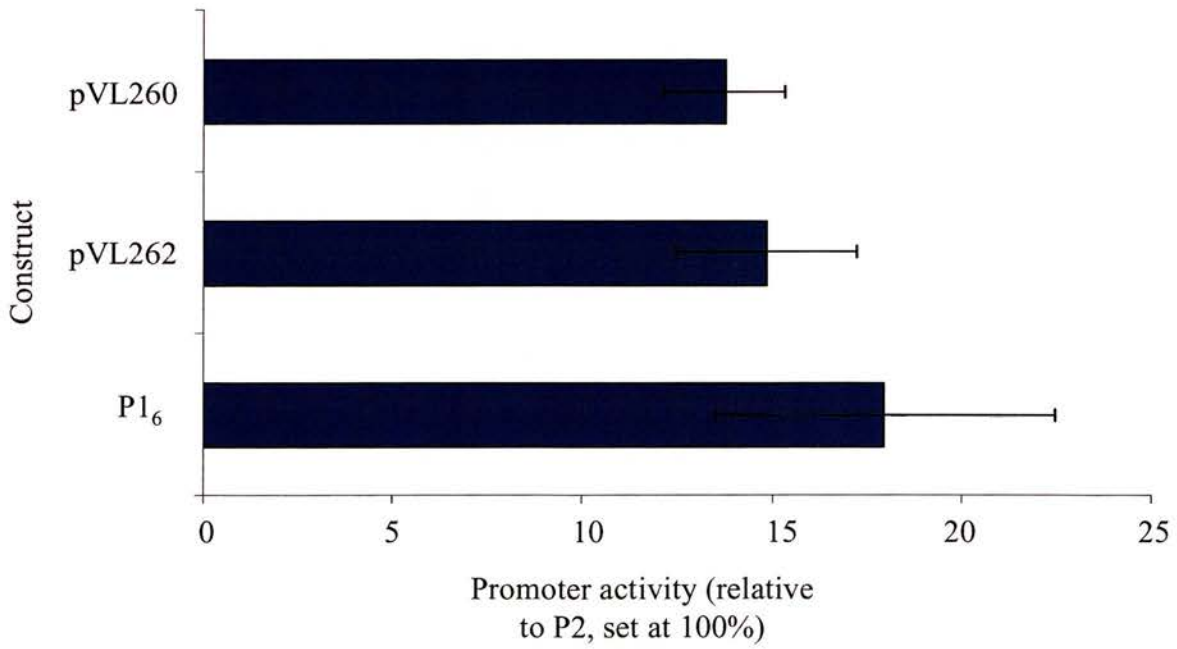
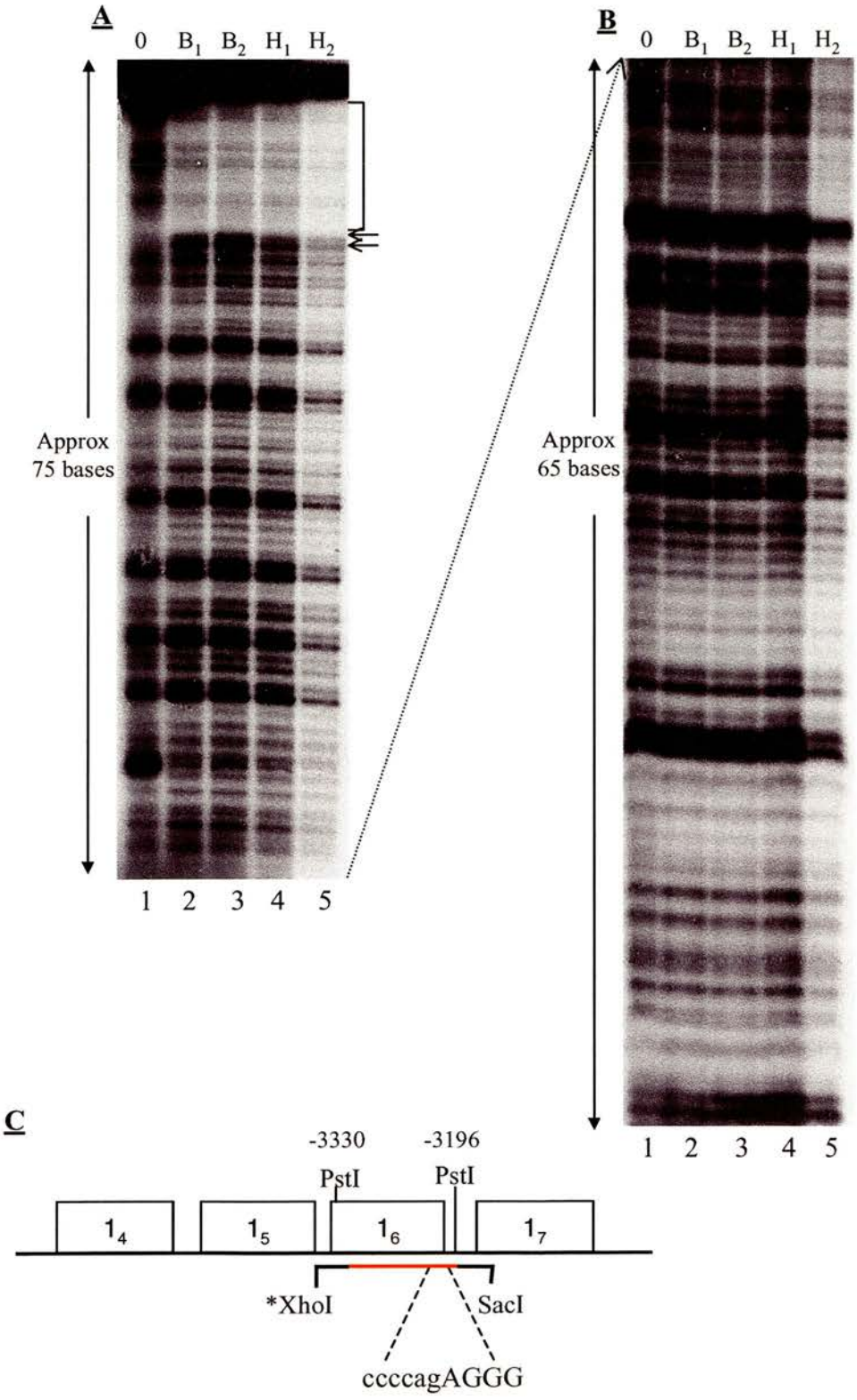

pVL260
pVL262
P1 6
Construct
0
5
10
15
20
25
Promoter activity (relative
to P2, set at 100%)

Figure 4.8 *B103 and H4IIE nuclear extracts protect a 'footprint' on the 134bp PstI fragment from DNaseI digestion.*
DNaseI protection analysis of the 134bp *PstI* fragment F_B, using B103 and H4IIE nuclear extracts. A and B represent successive loadings of the same reactions; the dotted arrow indicates the continuation of the sequence. Lane 1, no added protein; lane 2, 10μg B103 extract (B_1); lane 3, 50μg B103 extract (B_2); lane 4, 8μg H4IIE extract (H_1); lane 5, 48μg H4IIE extract (H_2). Brackets indicate the region of DNA protected from *DNaseI* digestion. Arrows indicate nucleotides hypersensitive to *DNaseI*.
C: Diagram of the GR promoter showing the F_B fragment including the 134bp PstI fragment (shown in red) and the short linker sequences (shown in black) present in the vector. The enzymes used to generate F_B are shown; the radiolabelled end is marked with '*'. The location and sequence of the footprint is also shown. Capital letters indicate a core part of a putative AP2 binding site.


A
0 B1 B2 H1 H2
Approx
75 bases
1 2 3 4 5
B
0 B1 B2 H1 H2
Approx
65 bases
1 2 3 4 5
C
-3330
-3196
PstI
PstI
1 4
1 5
1 6
1 7
*XhoI
SacI
ccccagAGGG

The F_A fragment, which was radiolabelled at the opposite end and on the opposite strand to F_B, was used to determine the extent of the footprint on the other strand. However, results of 2 independent experiments showed a weak footprint at the corresponding site that was only detectable by densitometric analysis (fig 4.9). The integrity of B103 and H4IIE nuclear extracts was controlled for in parallel experiments in which FP1 was detected on the F_B fragment.

4.3.2.2 B103 extract, but not recombinant AP2 protein, protects a 'footprint' on the 134bp *PstI* fragment from *DNaseI* digestion.

To determine if AP2 protein was indeed binding to FP1, further *DNaseI* protection analysis of the F_B fragment was carried out using B103 nuclear extract and recombinant AP2 protein. However, while results confirmed the formation of FP1 with B103 extract, AP2 protein failed to induce the same region of protection (fig. 4.10).

4.3.2.3 No *DNaseI* footprint is formed on the 274bp *PstI/BglI* fragment of the GR promoter with B103 or H4IIE nuclear extracts.

To further investigate protein-DNA interactions over the exon 1_7 region, *DNaseI* protection analysis was carried out using the F_C fragment. F_C represents the 274bp *PstI/BglI* region immediately downstream from the 134bp *PstI* fragment (fig. 4.11B). As such, F_A and F_C together comprise the region present in construct $P1_{7b}$, which showed high promoter activity in B103 cells. No detectable regions of *DNaseI* protection were observed on the F_C fragment (fig. 4.11A, B). Again, the integrity of B103 and H4IIE nuclear extracts was positively controlled for in parallel experiments in which a footprint was observed on the F_B fragment.

Figure 4.9 *No DNaseI 'footprint' is seen on the complementary strand of the 134bp PstI fragment with B103 or H4IIE nuclear extracts.*
A. *DNaseI* protection analysis of the complementary 134bp *PstI* fragment F_A, using B103 and H4IIE nuclear extracts. Lane 1, G+A Maxam & Gilbert sequencing reaction; lane 2, no added protein; lane 3, 10μg B103 extract (B_1); lane 4, 50μg B103 extract (B_2); lane 5, 8μg H4IIE extract (H_1); lane 6, 48μg H4IIE extract (H_2). Dashed brackets indicate the area complementary to the footprinted region observed on F_B.
B: Diagram of the GR promoter showing the F_a fragment including the 134bp PstI fragment (shown in red) and the short linker sequences (shown in black) present in the vector. The enzymes used to generate F_A are shown; the radiolabelled end is marked with '*'. The location and sequence of the putative footprint is also shown.

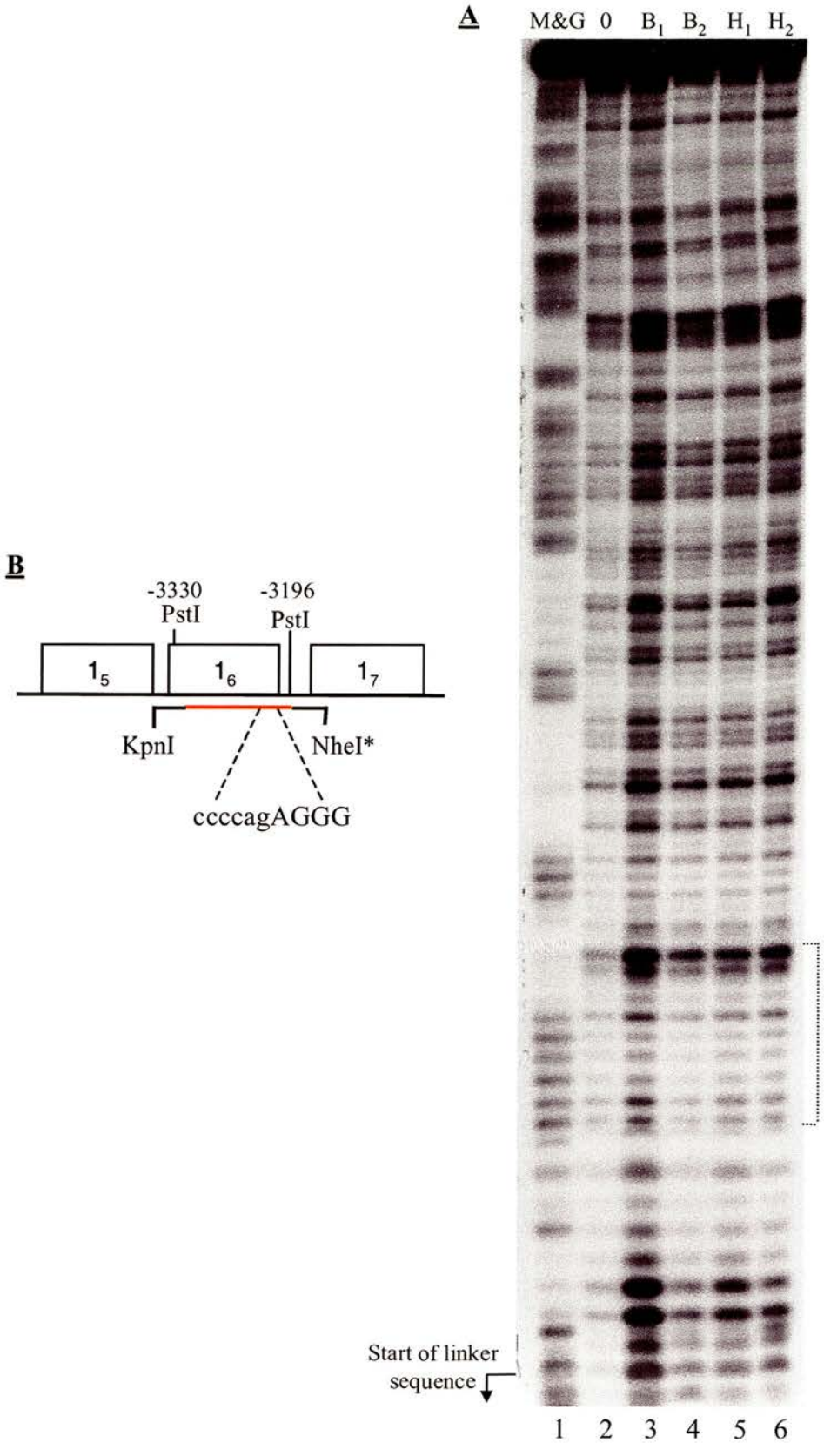

A
M&G 0 B1 B2 H1 H2
B
-3330
PstI
-3196
PstI
1 5
1 6
1 7
KpnI
NheI*
ccccagAGGG
Start of linker sequence
1 2 3 4 5 6

Figure 4.10 *B103 extract, but not recombinant AP2 protein, protects a 'footprint' on the 134bp PstI fragment from DNaseI digestion.*
DNaseI protection analysis of the 134bp *PstI* fragment F_B, using B103 nuclear extract and recombinant AP2 protein. A, B and C represent successive loadings of the same reactions. Lane 1, no added protein; lane 2, 10μg B103 extract (B_1); lane 3, 50μg B103 extract (B_2); lane 4, 3.8μg AP2 protein (A_1); lane 5, 7.6μg AP2 protein (A_2). Brackets indicate regions of DNA protected from *DNaseI* digestion. Arrows indicate nucleotides hypersensitive to *DNaseI*.

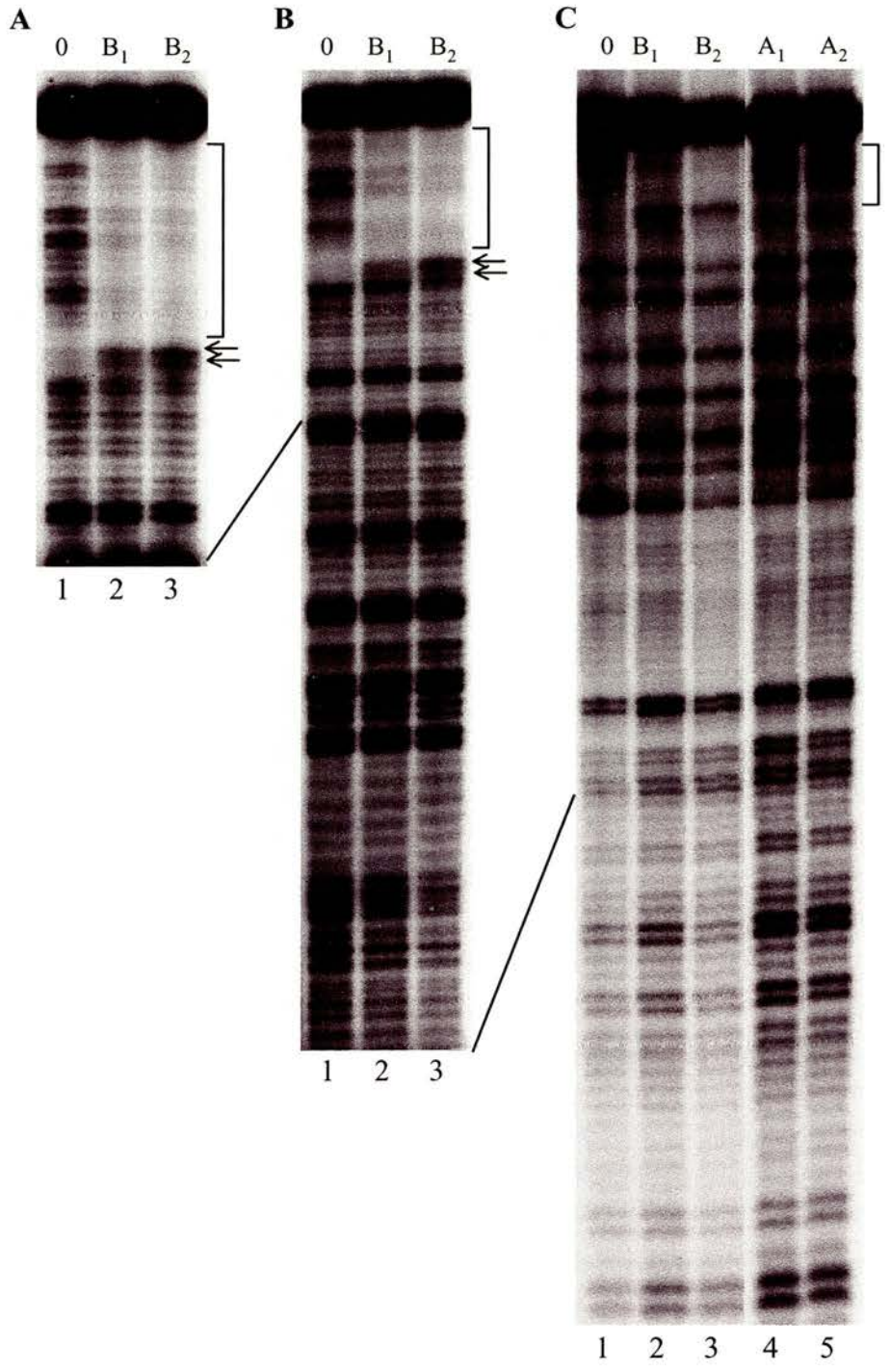
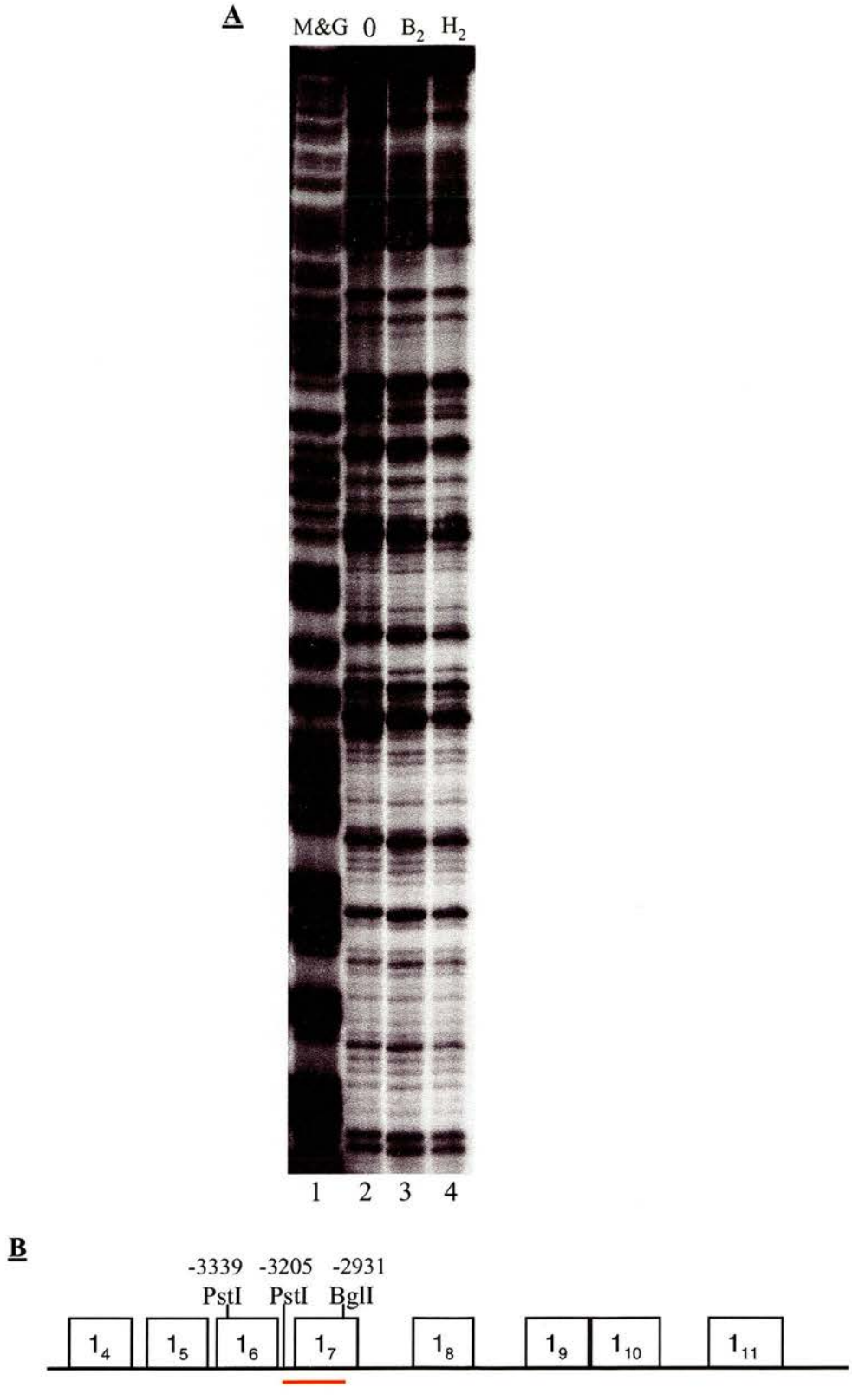

A
0 B1 B2
1 2 3
B
0 B1 B2
1 2 3
C
0 B1 B2 A1 A2
1 2 3 4 5

Figure 4.11 *No DNaseI 'footprint' is seen on the 274bp PstI/BglI fragment of the GR promoter with B103 or H4IIE nuclear extracts.*
DNaseI protection analysis of the 274bp *PstI/Bgll* fragment F_C, using B103 and H4IIE nuclear extracts. A: Lane 1, G+A Maxam & Gilbert sequencing reaction; lane 2, no added protein; lane 3, 75μg B103 extract (B_2); lane 4, 25μg H4IIE extract (H_2). B: Diagram of the GR promoter showing the location of the F_c fragment.


A
M&G 0 B_2 H_2
1 2 3 4
B
-3339 -3205 -2931
PstI PstI BglI
1_4 1_5 1_6 1_7 1_8 1_9 1_{10} 1_{11}

4.3.2.4 Factors in B103 and H4IIE nuclear extracts bind to the 134bp *PstI* fragment.

Further to the DNaseI footprinting experiments described above, protein-DNA interactions involving the 134bp PstI fragment were investigated by electrophoretic mobility shift assays (EMSAs). Factors present in both B103 and H4IIE cell extracts were shown to bind to the F_B fragment, with complex formation roughly proportional to nuclear extract concentration (fig 4.12). Three complexes were formed between the F_B fragment and B103 nuclear extract, with a further complex observed with H4IIE nuclear extract. Based on this data, nuclear extract concentrations of 25μg and 15μg for B103 and H4IIE, respectively, were used in further EMSAs.

Specificity of H4IIE and B103 nuclear protein binding to the 134bp *PstI* fragment was investigated by competitive EMSA. Using both extracts, a 100-fold excess of unlabelled F_B fragment successfully competed for binding to the labelled F_B probe at the upper 2 complexes, while the lower complexes remained unaffected (fig. 4.13; compare lanes 4 and 12 with 2 and 10). In contrast, a 100-fold excess of unlabelled non-specific competitor, F_{NS}, did not compete with the labelled probe for binding at any complex (fig. 4.13; compare lanes 6 and 14 with 2 and 10). It appears, therefore, that B103 and H4IIE protein binding at the upper 2 complexes is sequence specific, while binding at the lower complexes is not.

4.3.2.5 Factors in B103 and H4IIE nuclear extracts, including a Sp1-related protein, form specific complexes with the 134bp *PstI* fragment.

Supershift analysis, in which antibodies against candidate transcription factors were included in EMSAs, were used to identify the protein(s) responsible for specific binding of the 134bp *PstI* fragment. The Sp1 antibody caused a supershift in the DNA-protein complex formed with both B103 and H4IIE nuclear extracts (fig. 4.13; compare lanes 7 and 15 with lanes 2 and 10). However, it should be noted that not all the protein-DNA complex formed with either B103 or H4IIE extract was supershifted with the addition of the Sp1 antibody. In contrast, no supershift was

Figure 4.12 *Factors in B103 and H4IIE nuclear extracts bind to the 134bp PstI fragment.*
EMSA using the 134bp *PstI* fragment, F_B, and either B103 or H4IIE nuclear extracts. 30fmol [^{32}P]-labelled F_B was incubated with 2μg (lanes 2,7), 5μg (lanes 3,8), 10μg (lanes 4,9), 20μg (lanes 5,10) or 40μg (lanes 6,11) B103 or H4IIE extract. Lane 1 contained no nuclear extract. Filled arrows indicate protein-DNA complexes.

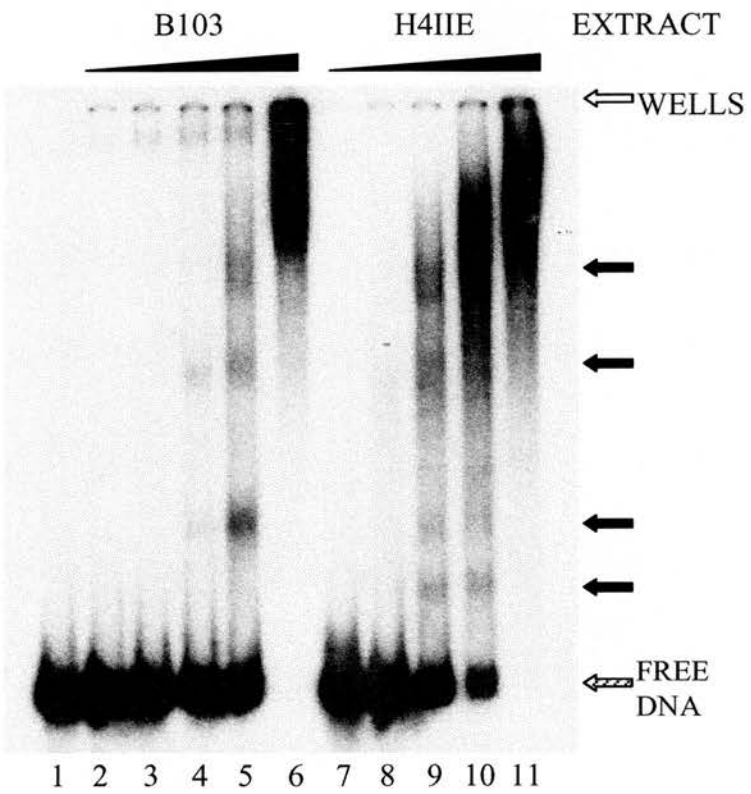
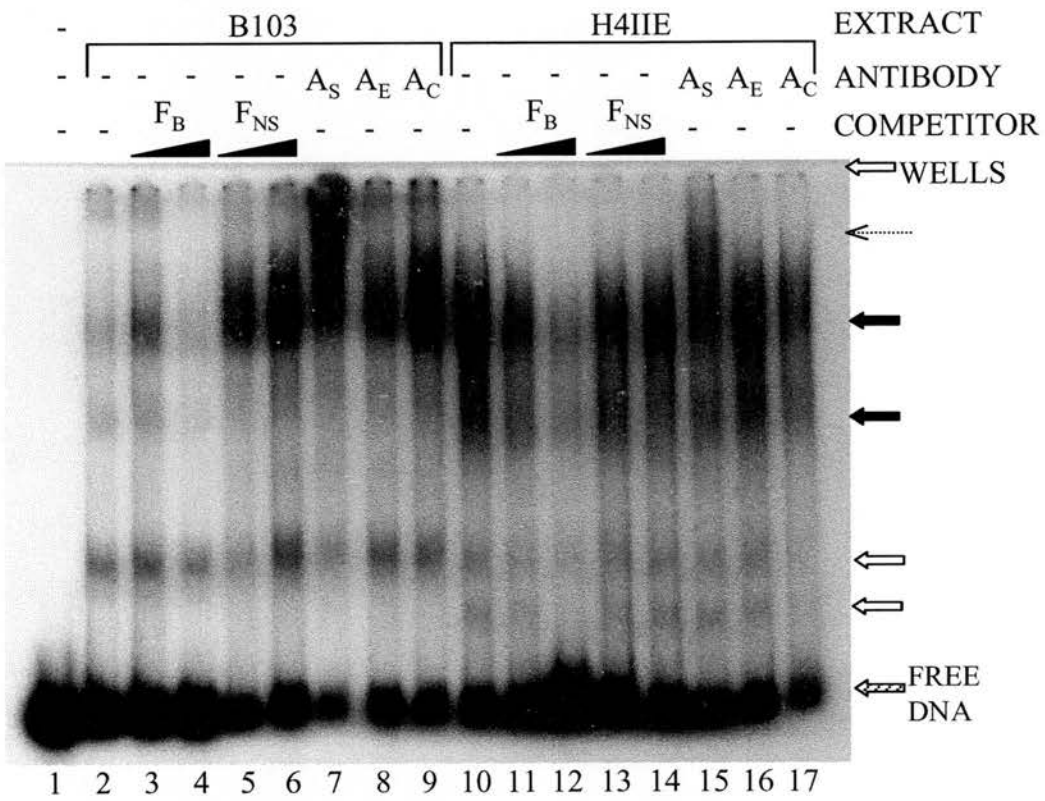

B103
H4IIE
EXTRACT
WELLS
FREE
DNA
1 2 3 4 5 6 7 8 9 10 11

Figure 4.13 *Factors in B103 and H4IIE nuclear extracts, including a Sp1-related protein, form specific complexes on the 134bp PstI fragment.*
EMSA using the 134bp *PstI* fragment, F_B, and either B103 or H4IIE nuclear extracts. 30fmol [^{32}P]-labelled F_B was incubated with 25μg B103 or 15μg H4IIE nuclear extract in the absence or presence of 10- or 100-fold molar excess of unlabelled specific competitor DNA F_B (lanes 2,3,11,12) or unlabelled non-specific competitor DNA F_{NS} (lanes 5,6,13,14), or in the presence of 1μg antibody against Sp1(A_S), Egr1(A_E) or Phospho-Stat1 (A_C). Lane 1 contained no nuclear extract. Filled and un-filled arrows indicate specific and non-specific protein-DNA complexes respectively. A dotted arrow indicates a super-shifted complex.

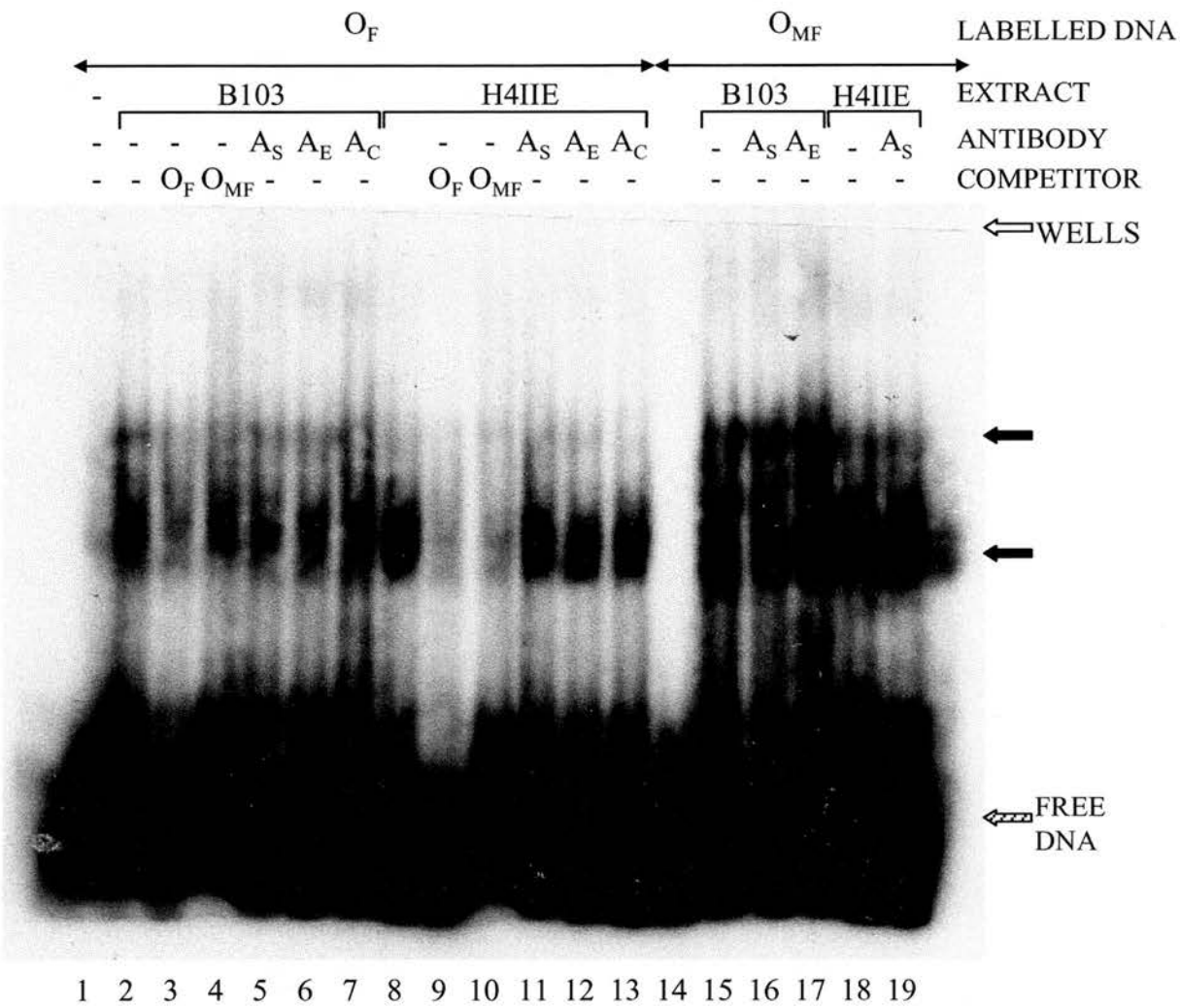

B103
H4IIE
EXTRACT
ANTIBODY
COMPETITOR
A_S A_E A_C
F_B F_{NS}
WELLS
FREE DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

observed following the inclusion of antibodies against either Egr-1 (fig 4.13; lanes 8 and 16) or the negative control Phospho-Stat1 (fig 4.13; lanes 9 and 17). Results suggest, therefore, that Sp1 or an antigenically-related protein present in both B103 and H4IIE extracts binds to the F_B fragment.

4.3.2.6 Factors in B103 and H4IIE nuclear extracts bind to an oligonucleotide corresponding to the 'footprinted' region of the 134bp *PstI* fragment.

The *DNaseI* footprinting data described in 4.3.1.1 showed binding at FP1 with both B103 and H4IIE nuclear extracts. In order to confirm this, EMSAs were performed using a radiolabelled synthetic oligonucleotide, O_F, representing the sequence protected in FP1. In addition, a further labelled oligonucleotide, O_{MF}, in which the putative AP2 binding site was abolished (see section 2.1.7.3), was also used. Figure 4.14 shows that factors present in B103 extract formed two complexes with O_F, with binding at the lower complex being more intense than at the upper. The same two complexes were formed between H4IIE extract and O_F, although the upper complex is even less intense in this case. Analysis of the gel also suggests the possibility of binding at a further upper complex, although longer exposure of the gel did not increase the intensity of these bands. A 75-fold molar excess of unlabelled O_F successfully competed with the labelled O_F probe for binding at the two lower complexes formed with B103 extract, while the same excess of unlabelled O_{MF} did not. In contrast, an excess of both unlabelled O_F and O_{MF} oligonucleotides successfully competed with the labelled O_F probe for binding to H4IIE extract. However, it should be noted that the same complexes were also formed between factors present in both B103 and H4IIE nuclear extracts and the mutant oligonucleotide, O_{MF}. Furthermore, antibodies against Sp1, Egr-1 and the negative control, Phospho-Stat1, did not induce a supershift in either labelled oligonucleotide or with either nuclear extract.

Figure 4.14. *Factors in B103 and H4IIE nuclear extracts bind to an oligonucleotide corresponding to the 'footprinted' region of the 134bp PstI fragment.*
EMSA using O_F and O_{MF} oligonucleotides (corresponding to the correct and mutated sequence of the footprinted region of the 134bp *PstI* fragment respectively) and B103 or H4IIE nuclear extract. 0.1pmol [^{32}P]-labelled O_F or O_{MF} was incubated with 25μg B103 or 15μg H4IIE nuclear extract in the absence or presence of 75-fold molar excess of unlabelled O_F (lanes 3,9) or O_{MF} (lanes 4,10), or in the presence in 1μg Sp1(A_S), Egr1(A_E) or Phospho-Stat1 (A_C) antibodies. Solid arrows indicate protein-DNA complexes. The faint bands observed at the top of the gel did not increase in intensity upon longer exposure (data not shown).


O_F
O_{MF}
LABELLED DNA
B103
H4IIE
B103
H4IIE
EXTRACT
A_S A_E A_C
A_S A_E A_S
ANTIBODY
O_F O_{MF}
O_F O_{MF}
COMPETITOR
WELLS
FREE DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

4.3.2.7 Factors in B103 and H4IIE nuclear extracts bind to an oligonucleotide encoding a consensus Sp1 binding site.

Further to the supershift of complexes between the F_B fragment and B103 and H4IIE extract induced by an antibody against Sp1 (fig 4.13), EMSAs were carried out using radiolabelled oligonucleotides containing either a consensus (O_S) or mutant (O_{MS}) Sp1 binding site. Both B103 and H4IIE nuclear proteins formed three complexes with O_S (figure 4.15; lanes 2-11), but only two with O_{MS} (figure 4.15; lanes 13-16). With both cell extracts, protein binding at the upper complex was successfully competed by a 60-fold excess of O_S (figure 4.15; lanes 5 and 10), but not by a 60-fold excess O_{MS} (figure 4.15; lanes 6 and 11). It therefore appears that binding between either cell extract and O_S at the upper complex is specific, while binding at the lower complexes is non-specific.

The inclusion of a Sp1 antibody induced a weak supershift in the DNA-protein complex formed with both B103 and H4IIE nuclear extracts (figure 4.15; lanes 3 and 8). In addition, the presence of Sp1 antibody caused the intensity of the shifted complex to decrease relative to the zero antibody reactions (figure 4.15; lanes 2 and 7). Together these observations provide evidence of sequence-specific Sp1 or Sp1-related protein binding activity in both cell extracts.

4.3.2.8 Factors in B103 and H4IIE nuclear extracts bind to oligonucleotides encoding both consensus and mutant AP2 binding sites.

Although *DNaseI* protection analyses failed to show binding of recombinant AP2 protein at FP1, results were confirmed by EMSAs using radiolabelled oligonucleotides containing either a consensus (O_A) or mutant (O_{MA}) AP2 binding site. O_A formed one complex with B103 nuclear extract, while two complexes were formed with H4IIE nuclear extract (fig.4.16). Using B103 extract, an excess of unlabelled O_A successfully out-competed the labelled O_A probe for binding. This was also the case for H4IIE, where competition for binding at the upper complex was stronger than at the lower. In addition, unlabelled O_{MA} successfully competed for

Figure 4.15. *Factors in B103 and H4IIE nuclear extracts bind to an oligonucleotide encoding a consensus Sp1 binding site.*
EMSA using oligonucleotides encoding consensus (O_S) and mutant (O_{MS}) Sp1 binding sites and either B103 or H4IIE nuclear extracts. 0.1pmol [^{32}P]-labelled O_S or O_{MS} was incubated with 25μg B103 or 15μg H4IIE nuclear extract in the absence or presence of 60-fold molar excess of unlabelled O_S (lanes 5,10) or O_{MS} (lanes 6,11), or in the presence of 1μg Sp1(A_S) or Phospho-Stat1 (A_C) antibodies. Filled and un-filled arrows indicate specific and non-specific protein-DNA complexes respectively. A dashed arrow indicates a super-shifted complex. A longer exposure of the bracketed region containing the shifted complexes is also shown.

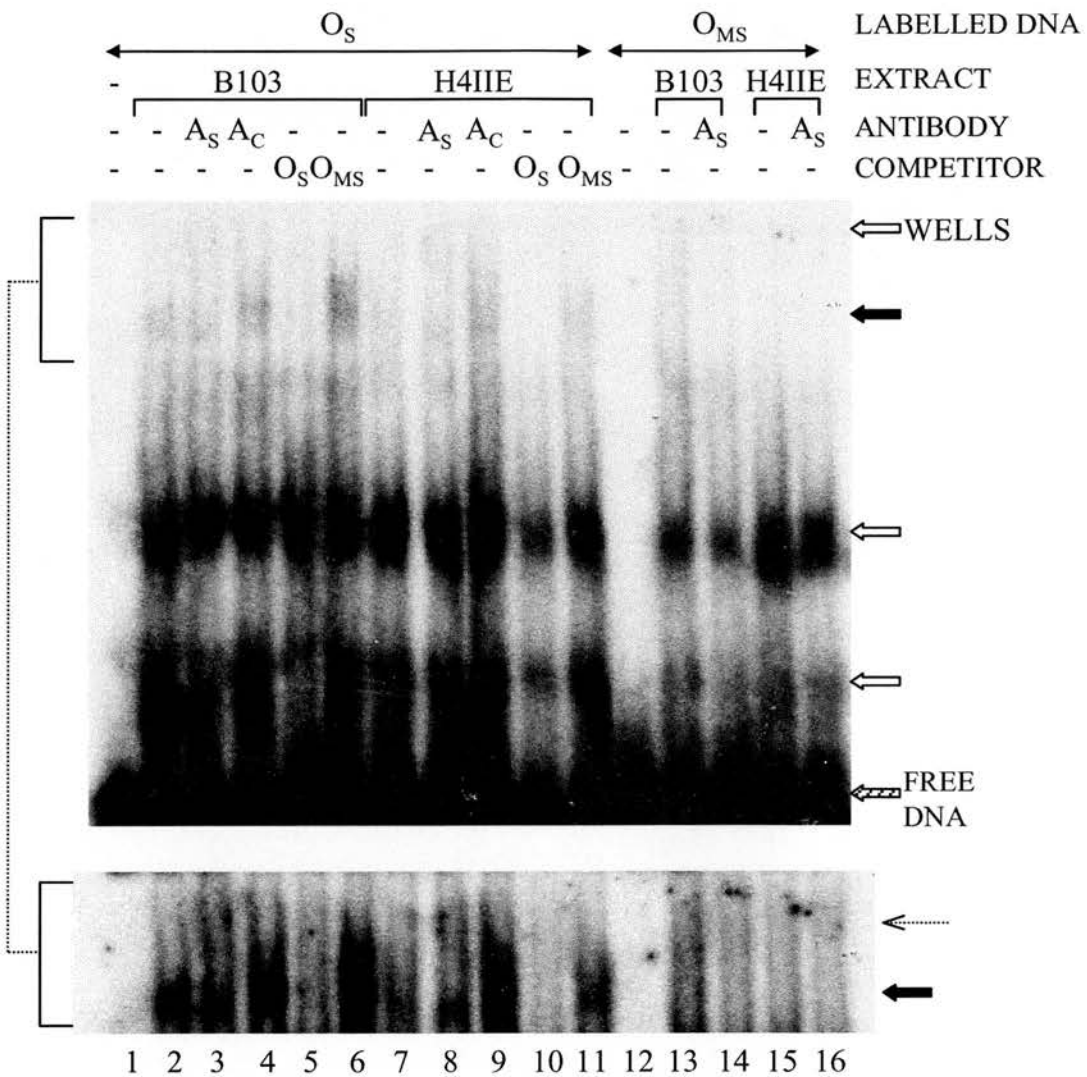
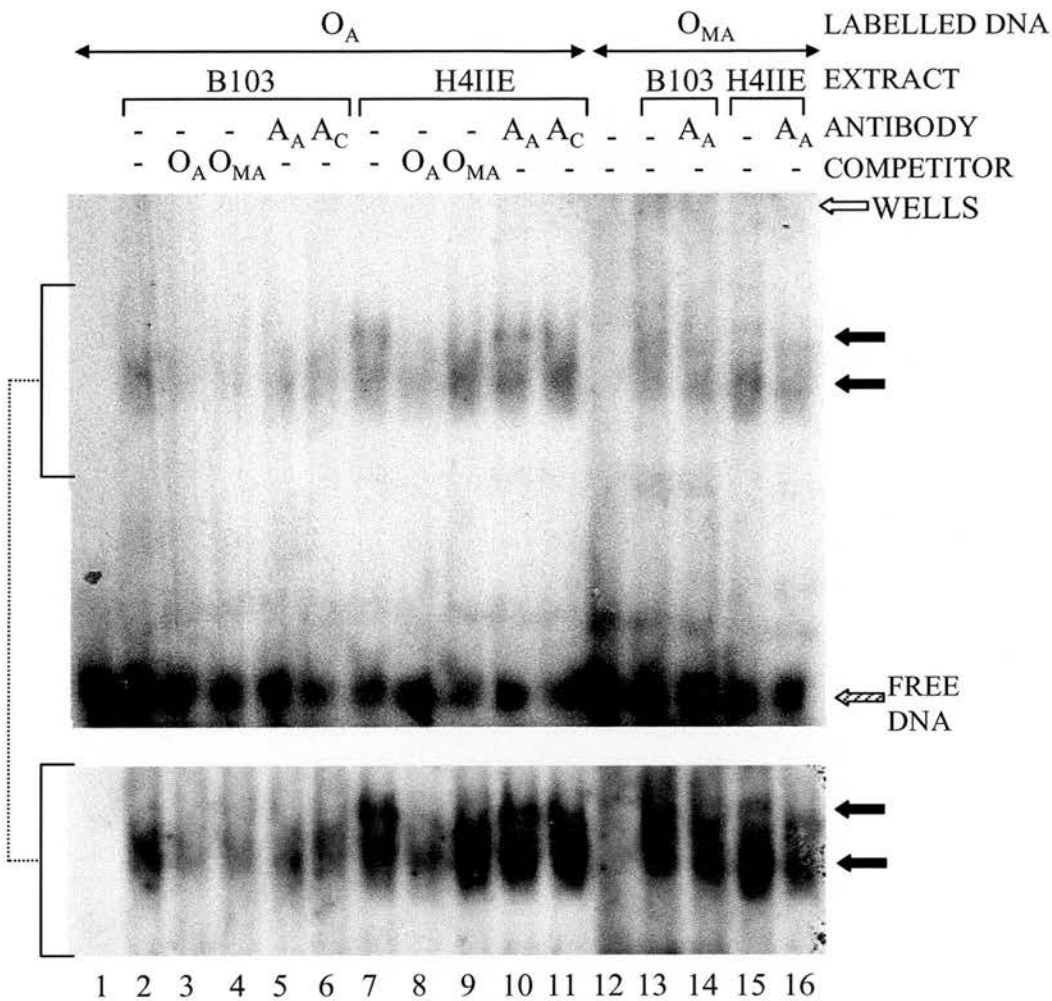

O_S
O_{MS}
LABELLED DNA
B103
H4IIE
B103
H4IIE
EXTRACT
A_S A_C
A_S A_C
A_S
A_S
ANTIBODY
O_S O_{MS}
O_S O_{MS}
COMPETITOR
WELLS
FREE DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4.16 *Factors in B103 and H4IIE nuclear extracts bind to oligonucleotides encoding both consensus and mutant AP2 binding sites.*
EMSA using oligonucleotides encoding consensus (O_A) and mutant (O_{MA}) AP2 binding sites and either B103 or H4IIE nuclear extracts. 0.1pmol [^{32}P]-labelled O_A or O_{MA} was incubated with 25μg B103 or 15μg H4IIE nuclear extract in the absence or presence of 60-fold molar excess of unlabelled O_A (lanes 3,8) or O_{MA} (lanes 4,9), or in the presence in 1μg AP2 (A_A) or Phospho-Stat1 (A_C) antibodies. Solid arrows indicate specific protein-DNA complexes. A longer exposure of the bracketed region containing the shifted complexes is also shown.


O_A
O_{MA}
LABELLED DNA
B103
H4IIE
B103
H4IIE
EXTRACT
- - - A_A A_C - - - A_A A_C - - A_A - A_A
ANTIBODY
- O_A O_{MA} - - - O_A O_{MA} - - - - - - - -
COMPETITOR
WELLS
FREE DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

binding at the B103/O_A complex, but this was not the case for either of the two H4IIE/O_A complexes. However, once again, complexes were formed between both B103 and H4IIE nuclear extracts and the mutant oligonucleotide, O_{MA}. The inclusion of an AP2 antibody did not induce a significant supershift or reduction in intensity of the protein-DNA complex using either O_A or O_{MA} with either cell extract.

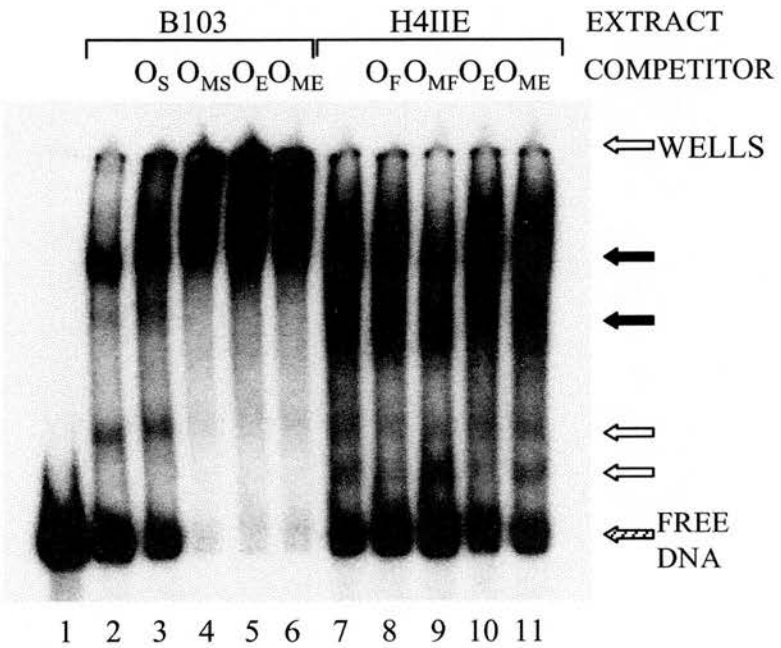
4.3.2.9 Binding between B103 and H4IIE nuclear extracts and F_B is not competed by either Sp1 or Egr1 consensus oligonucleotides.

To further investigate binding specificity of the F_B fragment, competition EMSAs were carried out using unlabelled oligonucleotides representing Sp1 (O_S and O_{MS}) and Egr1 (O_E and O_{ME}) binding sites as well as FP1 (O_F and O_{MF}). Complexes formed between H4IIE nuclear extract and F_B were not competed by a 30-fold molar excess of unlabelled O_F, O_{MF}, O_E or O_{ME} (fig.4.17). Similarly, complexes formed between B103 nuclear extracts and FB were not competed by an excess of unlabelled O_S. However, there was a loss of free DNA in reactions including B103 extracts and excesses of O_{MS}, O_E and O_{ME}. The reason for this is unclear, although it appears that a portion of the complexes remained in the well during electrophoresis. As such, it is difficult to assess if O_{MS}, O_E and O_{ME}, do indeed compete for binding between B103 extracts and F_B, although results using H4IIE extract and those shown in fig 4.15 suggest this is unlikely.

4.4 Discussion

The experiments described in this chapter were designed to (i) explore the hypothesis that alternate GR exons 1 are driven by individual promoters showing tissue-specific activity and (ii) further investigate elements of the CpG island region involved in GR regulation. With regard to objective (i), transient transfections using the 3' deletion series of P2, which measures the promoter activity associated with each individual exon 1, showed that $P1_7$ was the only construct to show tissue-specific activity. Specifically, $P1_7$ activity was higher in B103 (rat neuroblastoma) and C6 (rat glioma) cells than in H4IIE (rat hepatoma) or HepG2 (human hepatoma) cells. Experiments

Figure 4.17 *Binding between B103 and H4IIE nuclear extracts and F_B is not competed by either Sp1 or Egr1 consensus oligonucleotides.*
EMSA using 134bp *PstI* fragment, F_B, and either B103 or H4IIE nuclear extracts. 30fmol [^{32}P]-labelled F_B was incubated with 25μg B103 or 15μg H4IIE nuclear extract in the absence or presence of 30-fold molar excess of unlabelled consensus Sp1 oligonucleotide O_S (lane 3), mutant Sp1 oligonucleotide O_{MS} (lane 4), consensus Egr-1 oligonucleotide O_E (lanes 5,10), mutant Egr-1 oligonucleotide O_{ME} (lanes 6,11), or the 134bp *PstI* fragment 'footprinted' oligonucleotides O_F and O_{MF} (lanes 8,9). Based on data shown in fig 4.12, filled and un-filled arrows indicate specific and non-specific protein-DNA complexes respectively.


B103
H4IIE
EXTRACT
O_S O_{MS} O_E O_{ME}
O_F O_{MF} O_E O_{ME}
COMPETITOR
WELLS
FREE
DNA
1 2 3 4 5 6 7 8 9 10 11

using the 5' deletion series of $P1_7$ confirmed that a 134bp *PstI* fragment is necessary for high neural-specific activity. Deletion of this fragment from P2 caused a significant decrease in activity in B103, but not H4IIE cells, suggesting that the 134bp *PstI* fragment makes a significant contribution to overall P2 activity in neural cells only. The neural-specific high promoter activity of this fragment was found to be orientation-dependent. Under the accepted definition of an enhancer as 'a DNA element that increases transcription in a position- and orientation-independent manner' (Gill, 2001), the directionality of the 134bp *PstI* fragment rules out a classical enhancer role. In addition, the fragment did not activate a heterologous promoter in either orientation, confirming that this region does not show any enhancer properties. Instead, the fragment was shown to be an orientation-dependent promoter that was significantly more active in B103 than H4IIE cells.

DNaseI footprinting analysis of the 134bp *PstI* fragment (F_B) reproducibly identified a region near the 3' end of the fragment that was protected by both B103 and H4IIE nuclear extracts. Confusingly, *DNaseI* footprinting analysis of the same 134bp *PstI* fragment, labelled on the opposite strand (F_A), showed only very weak protection at the site corresponding to the previously identified footprint. The possible causes for this discrepancy is discussed in more detail later.

Sequence analysis showed the footprinted region to contain a putative AP2 transcription factor binding site. However, *DNaseI* footprinting analyses using recombinant AP2 protein did not demonstrate the same region of protection. While it should be noted that no positive control for the integrity of the AP2 protein was included, further evidence against a role for AP2 was provided by EMSAs. Specifically, while complexes were formed between factors present in both B103 and H4IIE nuclear extracts and a synthetic oligonucleotide containing FP1, an oligonucleotide in which the putative AP2 site present in FP1 was mutated was also shown to bind to both extracts. There are three possible explanations for this observation: (i) the putative AP2 site is not responsible for the observed binding; (ii) the mutation has created a new transcription factor binding site; or (iii) the binding is non-specific and occurs as a result of the high concentrations of nuclear extract used

(15-25μg). In addition, while both B103 and H4IIE nuclear extracts bound to an oligonucleotide containing a consensus AP2 binding site, complexes were also formed on an oligonucleotide in which the AP2 site was mutated, suggesting that either the complexes were non-specific or binding did not involve the mutated nucleotides. Furthermore, complexes formed between B103 or H4IIE extracts and the consensus AP2 nucleotide were not supershifted upon the inclusion of an antibody against AP2, suggesting that the observed complexes did not contain AP2 protein. The balance of evidence therefore suggests that AP2 binding is not responsible for FP1.

Initial sequence analysis of the 134bp *PstI* fragment showed it to encode an Egr-1 (synonymous with NGFI-A) transcription factor binding site. Previous work has also shown that increases in hippocampal GR mRNA following environmental enrichment of infant rats correlates with increased NGFI-A mRNA (Olsson et al., 1994; Meaney et al., 2000). The higher relative abundance of exon 1_7-containing transcripts in rat hippocampus and the high neural-specific promoter activity of the 134bp *PstI* fragment led to the hypothesis that up-regulation of NGFI-A following perinatal programming might cause an up-regulation of exon 1_7 transcription, consequently leading to an overall rise in GR mRNA levels (Meaney et al., 2000). However, *DNaseI* footprinting experiments and EMSAs using both B103 and H4IIE nuclear extracts showed no evidence of NGFI-A binding at its putative site or anywhere else on the fragment. Further results have suggested that, in this instance, the NGFI-A site responsible for up-regulation of GR is located further downstream from the 134bp *PstI* fragment (Meaney M., personal communication). This site resides in the region corresponding to F_C, although *DNaseI* footprinting studies carried out during this investigation did not show any evidence of binding between B103 nuclear extracts and the F_C fragment. However, on the basis of data presented here, the F_C complementary strand should also be tested.

Several pieces of evidence suggested that Sp1 or an Sp-1 related transcription factor, present in both H4IIE and B103 nuclear extracts, binds to the 134bp *PstI* fragment in a specific manner. Specifically, the inclusion of an antibody against Sp1 caused a

supershift in complexes formed between F_B and both H4IIE and B103 nuclear extracts. In addition, both H4IIE and B103 extracts were shown to form specific complexes with a synthetic Sp1 consensus oligonucleotide. However, it is notable that an excess of unlabelled consensus Sp1 oligonucleotide did not compete with the labelled F_B fragment for binding to B103 extract. Considering the GC-rich nature of the whole region, including the 134bp *PstI* fragment, perhaps evidence showing binding by Sp1 or a Sp-1 related protein was to be expected. As such, while Sp1 binding appears to be specific, whether this is at a high enough affinity to be relevant *in vivo* remains unconfirmed.

It should be noted that time constraints did not permit the repitition of several band shift experiments shown here. The specificity of potential binding over the 134bp *PstI* region should therefore be confirmed by further experiments in which consistent, rerproducible band shifts are observed.

It is interesting to note that the *DNaseI* footprinting experiments of the 134bp *PstI* fragment mirror the directionality observed in transient transfection studies; activity and binding of this fragment appear to be completely orientation-specific. Given that the physical properties of transcription factor binding protect both strands from *DNaseI* digestion, regardless of whether contacts are made on only one strand (Gill, 2001), the presence of a *DNaseI* footprint on only one strand of this fragment is puzzling. The integrity of B103 and H4IIE nuclear extracts used in experiments with F_A was controlled for in parallel experiments in which protection was observed on fragment F_B, ruling out degradation of protein as a source of the discrepancy. Recent studies have shown that protein-DNA binding is often extremely dynamic, whereby a binding protein continually 'hops' on and off its binding site (Becker et al., 2002; Maruvada et al., 2003). These experiments only capture a 'snapshot' of binding over the fragment and naturally the more dynamic the binding, the greater the difficulty in observing a footprint. It is therefore conceivable that interactions in this region may be too dynamic to leave a strong footprint, although it is unclear why binding should be more easily detectable on one strand than its complement.

However, it is conceivable that protection would only be seen on one strand if the sequence in question were 'unwound'. This would be the case if *RNA polymerase II* was bound and active on this fragment and would also explain the directionality of its promoter activity. If this region does indeed represent a region at which directional transcription is initiated, the presence of factors involved in the *RNA polymerase II* transcription machinery would be predicted. Unfortunately, EMSAs that included an antibody against TFIID, a protein complex known to be involved in the transcription initiation complex, showed no supershift in the protein-DNA complex with either H4IIE or B103 cell extracts (data not shown). However, TFIID is a large complex of proteins (Gill, 2001) and as such is relatively easily degraded. It may therefore be the case that the protein extracts used in the experiments described here might not have been of sufficient quality to maintain the integrity of the TFIID complex.

Significantly, the protein binding observed at the 134bp *PstI* fragment is not specific to the neural cell line B103, but is also seen with H4IIE (hepatoma) extract. As such, the experiments described here do not provide a candidate neural-specific protein whose binding accounts for the high neural-specific activity of this region. It may be the case that post-transcriptional mechanisms account for the high neural-specific activity of $P1_7$, $P1_{7a}$ and $P1_{7b}$. For instance, exon 1_7-associated transcripts may be more stable or more easily translated in neural cells. Similarly, if the region does indeed contain a transcription initiation site, it may be that the start site is simply more active in B103 than H4IIE cells, although the reason for this remains unclear.

With regard to objective (ii), transient transfection data provided some further insights into the functional elements involved in transcriptional activity of the GR promoter region. In all cell lines tested, P2, which represents the whole CpG island promoter region, showed the highest activity. The low promoter activity associated with most alternate exons 1 must therefore combine to produce high overall activity. To further investigate this, the total cumulative activity of all constructs in the 3' deletion series was calculated in the four cell lines (table 4.1). The values for B103, C6, HepG2 and H4IIE cells are 148%, 124%, 102% and 42% respectively. The

cumulative activity of all CpG island exon 1 constructs therefore accounts for at least all of P2 activity (set at 100%) in B103, HepG2 and C6 cells, but not in H4IIE cells. The fact that the cumulative activity of individual exons 1 exceeds that of P2 in B103 and C6 cells also suggests that there might be an upper limit to total (P2) transcription rate over the region. In addition, the apparent additive activity of individual exons 1 indicates that there is little 'promoter competition', i.e. initiation of transcription at one exon does not prevent initiation at another.

Cell Line	Cumulative promoter activity of 3' deletion constructs
B103	148%
C6	124%
HepG2	102%
H4IIE	42%

Table 4.1 *Combined promoter activity of the 3' deletion constructs.*
Table showing the combined promoter activity of transiently transfected constructs of the 3' deletion series ($P1_5$, $P1_6$, $P1_7$, $P1_8$, $P1_{9/10}$, $P1_{10}$ and $P1_{11}$). Values were calculated from the means of each transiently transfected construct in each cell line and are expressed as a percentage of total P2 activity (nominally set at 100%).

The difference between the cumulative activity of individual exons 1 and overall P2 activity in H4IIE cells is puzzling. It may be the case that, in the H4IIE line, the exons are working synergistically. Alternatively, the result may reflect the different technique used to transiently transfect H4IIE cells (cationic lipid method) compared to the other cell lines (calcium phosphate method). However, levels of P2 in relation to empty vector are not significantly different in H4IIE cells to those seen in B103 or C6 cells, which makes differences caused by transfection technique unlikely.

The lack of significant promoter activity associated with $P1_6$ and $P1_{10}$ was surprising given the predominance of exon 1_6 and 1_{10} containing transcripts in all tissues tested (Strahle et al., 1992; McCormick et al., 2000) Strahle *at al* used RPA analysis to carry out quantification of mouse GR transcripts 1B and 1C (corresponding to rat exons 1_6 and 1_{10} respectively), in mouse liver, brain, S49 (mouse T lymphoma) and WEHI-7 (mouse T lymphoma) cells (Strahle et al., 1992). They detected a comparatively stronger signal for 1B in liver relative to other cells and tissues, and

observed several 1C transcripts (indicating variable transcription start sites) which were present at varying levels in all samples. They therefore concluded that transcription of exons 1B and 1C is driven by individual promoters showing cell-specific activity. However, it should be noted that while 1B and 1C expression levels were compared between tissues, the pattern of 1B versus 1C expression within tissues was not shown. It may simply be the case that levels of exons 1B and 1C are both higher in mouse liver relative to other tissues and cells. In this way, exons 1B and 1C may not be differentially driven by tissue-specific promoters; rather transcription at both exons is simply higher in cells that express high levels of GR.

Data gained for $P1_6$ and $P1_{10}$ also contrast with evidence showing that luciferase reporter constructs containing sequences proximal to either human GR exons 1B or 1C (homologous to rat exons 1_6 and 1_{10} respectively) exhibited significant promoter activity in several human cell lines (Nobukuni et al., 1995; Breslin et al., 2001; Nunez & Vedeckis, 2002). Breslin *et al* showed no difference in the activity of promoter 1B between IM-9 (B-cell lymphoma), CEMC-7 (T-cell lymphoblast), Jurkat (human T), HeLa cells (human cervial carcinoma), with higher activity in WI-38 (fibroblast) cells than IM-9 cells but not significantly different to the other cell lines (Breslin et al., 2001). In contrast, there were significant differences in the activity of promoter 1C between all the cell lines (Breslin et al., 2001). However, similar to the case described above, the activity of promoter 1B and 1C constructs was compared between and not within tissues. In this way, the relative contribution of these promoters to activity within specific cell lines was not addressed.

Nunez *et al* have reported data showing that the 1B and 1C promoters exhibit differential activity within cell types (Nunez & Vedeckis, 2002). While there was no difference in activity between the two constructs in HeLa or Jurkat cells, the promoter activity of 1C was significantly higher than 1B in HepG2 cells (Nunez & Vedeckis, 2002). With regard to the 1B promoter, footprints have been reported at one GATA, three YY1 and four Sp1 transcription factor binding sites (Breslin & Vedeckis, 1998) (Nunez & Vedeckis, 2002). In addition, the relative contribution of these binding sites has been shown to be tissue-dependent. Specifically, deletion of

the three YY1 sites has been shown to lead to a decrease in promoter activity in HepG2 and Jurkat cells, but not in HeLa cells (Nunez & Vedeckis, 2002). Conversely, deletion of all four Sp1 sites caused a significant down-regulation in promoter activity in HeLa cells, but not in HepG2 or Jurkat cells (Nunez & Vedeckis, 2002). However, it should be noted that the resolution of the *DNaseI* protection data presented by Nunez *et al*, in which they claim to identify four footprints corresponding to one GATA and three Sp1 transcription factor binding sites, is relatively poor (Nunez & Vedeckis, 2002). Similarly, in several of the EMSAs, lanes corresponding to 'labelled fragment only' are empty; thus preventing comparison between DNA alone and potentially shifted complexes. Furthermore, addition of an antibody against GATA-3 failed to induce a supershift in the protein-DNA complex, and supershifts were only seen with the addition of an antibody against Sp1. Similar to the work presented here, it might be expected that evidence of Sp-1 binding might be observed at such a GC-rich sequence, particularly at high concentrations of nuclear extracts (Nunez *et al* do not report the concentration of HeLa extract used in their experiments). The physiological relevance of these Sp1 sites therefore remains to be seen.

With regard to promoter 1C, previous studies have identified five putative Sp1 sites, although their contribution to overall and tissue-specific GR regulation has not been investigated (Breslin & Vedeckis, 1998). In addition, Nobukuni *et al* reported the presence of three footprints on the 1C promoter, one of which was shown to bind AP2 in HepG2 cells (Nobukuni et al., 1995). Significantly, deletion of this AP2 site triggered a decline in promoter activity in HeLa and NIH3T3 cells, but not in the HepG2 cells in which binding had been observed (Nobukuni et al., 1995). The relevance of this transcription factor to GR regulation in HepG2 or indeed any other cell line therefore remains unconfirmed.

Despite the relative lack of clarity with regard to the transcription factors involved in regulating transcriptional activity over the GR promoter, particularly at sequences associated with exons 1B and 1C, the balance of evidence provides some interesting implications. Most significantly, it appears that tissue-specific GR expression is not

regulated by exclusively tissue-specific transcription factors. Instead, transcriptional activity is driven by ubiquitously expressed factors such as YY1 and Sp1. Furthermore, while deletion of the four YY1 sites from the whole human GR promoter resulted in a ~60% decrease in activity (Breslin & Vedeckis, 1998), their deletion from the isolated 1B promoter caused only a ~30% reduction (Nunez & Vedeckis, 2002). This finding suggests that the contribution made by these regulatory elements may be dependent upon their context within the whole promoter, i.e. there is inter-dependence between interactions at different regulatory elements. As such, isolation of these regulatory elements may not give an accurate picture of their overall contribution to promoter activity. If this is the case, then differences in the size and relative positions of fragments used in transient transfection assays may create marked differences in their activities. This may explain the apparent discrepancy between the promoter activities of the $P1_6$ and $P1_{10}$ constructs used in these experiments and the 1B and 1C constructs used by others.

This contextual dependence may also explain why the contribution of the 134bp *PstI* fragment is significantly higher within the $P1_{7b}$ construct than when isolated in a promoterless vector. Similarly, regulatory regions that are not present in the $P1_6$ and $P1_{10}$ constructs may contribute to the transcriptional activity at exons 1_6 and 1_{10}, hence the predominance of these GR mRNA isoforms in rat tissues (McCormick et al., 2000; McCormick, 2000). However, an alternative possibility, in which post-transcriptional mechanisms may act to specifically stabilise exon 1_6- and 1_{10}-containing GR mRNA isoforms, should also be mentioned. While transcriptional activity at each exon 1 is relatively similar in all cells, transcripts containing specific 5' leader sequences may be subject to differential, tissue-specific degradation, thus giving rise to characteristic patterns of GR mRNA isoforms, in which exon 1_6- and 1_{10}-containing transcripts predominate. This could be investigated by assaying the rate of degradation of transcripts containing alternate 5' leader sequences. Indeed, GR/GFP minigene constructs have already been synthesised in this laboratory and could be used for this purpose.

It is interesting that further investigation of the elements involved in regulating activity of the rat GR promoter supported this idea of contextually dependent regulatory regions. Experiments using the 5' deletion series identified two regions, R_1 (-4878 to -3912), and R_2 (-2806 to -2535), that together accounted for 80% of total P2 activity in B103 cells. R_1 incorporates exon 1_4 while R_2 includes exons1_8 and 1_9. Interestingly, the –3912 to –2806 deletion, which includes exons 1_5, 1_6 and 1_7, appears to have no effect on promoter activity. As such, the loss of the region represented by $P1_7$, which has previously been shown to be highly active in B103 cells, does not produce any further decrease in activity over and above that occurring as a result of the –4878 to –3912 deletion. This suggests that the contribution of the 1_7 promoter element, specifically the 134bp *PstI* fragment, may be relatively small in the context of the whole promoter. Consistent with this, while the 134bp *PstI* fragment was important in the context of $P1_{7b}$, its deletion from P2 resulted in a significant, but relatively small decrease in overall promoter activity.

The position-dependent nature of regulatory elements in the GR promoter is also supported by comparison of results in B103 cells for pVL262 (an internal *PstI* deletion construct), $P1_6$ (from the 3' deletion series) and pVL260 (from the 5' deletion series). The activity of two regions from –4878 to –3339 and –1770 to -9 can be assessed by comparison of data for these three constructs. Interestingly, there is no significant difference in activity between any of the constructs. In addition, the 5' deletion series showed that the –4878 to –3912 region accounted for 40% of P2 activity, although pVL262, in which –4878 to –3339 is present, is not more active than pVL260, in which it is absent. Regional promoter activity therefore appears to be dependent upon correct positioning within the whole promoter.

To conclude, the $P1_7$ construct was unique in showing tissue-specific promoter activity in neural cells. This result complements previous data showing the presence of 1_7–containing GR transcripts in rat hippocampus (McCormick et al., 2000). A 134bp *PstI* fragment was shown to be responsible for high neural-specific activity, although no neural specific protein-DNA interactions were observed over the region. Therefore, with the exception of 1_7, it appears that the alternate exons 1 that are

located in the CpG island region of the rat GR promoter are not driven by individual tissue-specific promoters. As such, regulatory elements appear not to be associated with individual exons 1 but are instead located over the whole GR promoter region. Furthermore, these elements do not appear to operate in isolation but are inter-dependent upon each other. Hence, the activity of regional promoter elements is dependent upon their correct positioning within the whole GR promoter region. Together with evidence showing that all exons 1 residing within the CpG island region are expressed in all tissues, it appears that tissue-specific regulation of GR mRNA levels does not primarily occur through the selective regulation of transcriptional activity at individual exons 1.

5 Investigation of the roles of exon 1-associated splice donor sites and chromatin structure in rat GR regulation.

5.1 Introduction & Aims

The work described in the previous two chapters investigated the hypothesis that alternate exons 1 located in the rat GR CpG island region are associated with tissue-specific regulation. However, results showed that all alternate exons 1 tested were ubiquitously expressed, and only one exon (1_7) appeared to be associated with a tissue-specific promoter. The balance of evidence therefore suggests that the selective activity of alternate exons 1 does not provide a mechanism for the tissue-specific regulation of GR. In light of this, alternative roles of this unusual and highly conserved structure were investigated.

Results presented in chapter 4 indicated that relatively insignificant regional promoter activity over the CpG island region combines additively to generate high overall activity. This suggests that the multiple exon 1 structure of GR might play a role in ensuring the high constitutive expression of this important housekeeping gene. As such, transcription can be thought of as initiating from multiple preferential sites (giving rise to multiple exons 1), but selection of individual start sites is relatively unimportant. In this way, transcription initiation over the whole CpG island region might combine to generate high and constitutive GR expression.

Under this hypothesis, the role of the splice donor site would be minimal. If transcription is initiating indiscriminately over the GR CpG island region, splicing should simply occur at the most proximal suitable donor site. There are also some significant implications for chromatin structure. This hypothesis implies that the GR CpG island region occupies an extended area of transcriptional activity and, as such, should be accompanied by a corresponding region of 'open' chromatin. Thus, the aim of experiments described in this chapter was to elucidate the role of the alternate CpG island exons 1 in the constitutive regulation of GR. Specifically, the role of the splice donor site and the chromatin conformation of the region were investigated.

5.2 Experimental Design

5.2.1 Site-directed mutagenesis of splice donors sites at exons 1_6, 1_{10} and 1_{11} within P2.

The role of exon 1-associated splice donor sites in GR regulation was assessed using constructs in which various splice donor sites were abolished. Site-directed mutagenesis of P2 was used to engineer GT to AC mutations in the splice donor sites at exons 1_6, 1_{10} and 1_{11}, generating the constructs P2Mut1_6, P2Mut1_{10} and P2Mut1_{11} respectively. In addition, a GT to AC mutation at the exon 1_{10} splice donor site was engineered within P1_{11}, resulting in P1_{11}Mut1_{10}. Transient transfection analysis in B103 cells was undertaken to investigate the effect of the mutations on promoter activity.

The effect of each mutation on splicing activity was assessed by RT-PCR and sequence analysis of RNA extracted from transfected cells. To this end, each transient transfection was performed using six replicates, with three dishes used for promoter activity assays and three dishes used to prepare RNA. Following RT-PCR, reactions were performed using 5' primers specific for individual exons 1 (1_5, 1_6, 1_7, 1_{10} and 1_{11}) in conjunction with a common 3' primer complementary to luciferase (section 2.1.7.1). Predicted product sizes are given in table 5.1. Positive control reactions for each primer pair were carried out using RNA from cells transfected with intact P2 plasmid. In order to control for DNA contamination of RNA samples, negative control reactions, in which AMV Reverse Transcriptase enzyme was omitted from the reverse transcription step, were carried out for each RNA sample and primer pair.

Transcript	Predicted size (bp)
1_5	210
1_6	210
1_7	204
1_{10}	279
1_{11}	220

Table 5.1 *Predicted sizes of P2 alternate exon 1 RT-PCR products*

5.2.2 *DNaseI* hypersensitive site mapping of the GR promoter locus.

The chromatin structure at the GR promoter locus was investigated by *DNaseI* hypersensitive (HS) site mapping. This technique is based on the principle that chromatin in an 'open' conformation is more sensitive to enzymatic digestion by *DNaseI* than 'closed' chromatin. Specifically, relatively small areas of open chromatin, usually coinciding with active promoter or enhancer elements, give rise to sites that are hypersensitive to digestion by *DNaseI*. Nuclei preparations of selected cells or tissues are treated with increasing concentrations of *DNaseI*, genomic DNA is isolated and subsequently restricted using an enzyme yielding a fragment that encompasses the whole region under investigation. This is known as the 'parent' fragment and *DNaseI* digestion at sites of hypersensitivity generates smaller 'daughter' fragments. After Southern blotting of the digested DNA and hybridisation with a radiolabelled probe, the relative sizes of 'parent' and 'daughter' bands are calculated to map regions of hypersensitivity.

Nuclei were isolated from H4IIE (rat hepatoma) cells and digested with three different concentrations of *DNaseI* enzyme (30u/ml, 40u/ml and 50u/ml) for four different lengths of time (1, 2, 4 and 8 min). Genomic DNA was isolated before treatment with *HindIII* to give a 3641bp parent fragment encompassing the whole of the GR CpG island region. A 'zero' reaction, which was not exposed to *DNaseI,* was also retained. In addition, DNA purified from *DNaseI*-treated H4IIE nuclei was digested with *SalI,* generating a large 30.1kb parent fragment, which encompassed the previously mentioned 3641bp *HindIII* region. Southern blotting was carried out on both sets of digests using a radiolabelled 881bp *SphI/HindIII* probe (section 2.1.9).

5.3 Results

5.3.1 Mutagenesis of splice donor sites at exons 1_6, 1_{10} and 1_{11} in the P2 construct does not significantly decrease promoter activity.

The role of the exon 1-associated splice donor sites was investigated using constructs in which the splice donor sites at exons 1_6, 1_{10} and 1_{11} were mutated within the P2 plasmid. The resulting constructs (P2Mut1_6, P2Mut1_{10} and P2Mut1_{11} respectively) were transiently transfected into B103 (rat neuroblastoma) cells. Transient transfections of the P1_{11}Mut1_{10} construct, in which the exon 1_{10} splice donor site of the P1_{11} plasmid was mutated, were also carried out. None of the mutated P2 constructs show significantly different activity to intact P2 (fig 5.1). In addition, there was no significant difference in activity between P1_{11}Mut1_{10} and P1_{11}. Mutation of splice donor sites, including those at the most abundant transcripts 1_6 and 1_{10}, therefore appears to have no effect on GR promoter activity.

5.3.2 Longer transcripts are associated with GR exons 1 at which the splice donor sites are mutated.

The effect of each mutation on splicing activity was investigated using RT-PCR and sequence analysis. RT-PCR reactions were carried out on RNA extracted from B103 cells transfected with P2, P2Mut1_6, P2Mut1_{10} and P2Mut1_{11}, using 5' primers specific for individual exons 1 (1_5, 1_6, 1_7, 1_{10} and 1_{11}) in conjunction with a common 3' primer complementary to luciferase. Reactions using RNA from cells transfected with intact P2 clearly generated products of the predicted size corresponding to exons 1_5, 1_6, 1_7, 1_{10} and 1_{11} (fig. 5.2). No bands were observed in negative control reactions performed without reverse transcriptase (data not shown). The exon 1_{10} product was absent from RNA of cells transfected with P2Mut1_{10}, but all other exon 1 products were present at the predicted sizes. Interestingly, RT-PCR analysis of RNA from cells transfected with P2Mut1_6 showed a 290bp 1_6 product that was larger than predicted. Further RT-PCR analysis using the 1_6 primer produced an additional 476bp product (fig. 5.2B). The 290 and 476bp PCR products were each sub-cloned

Figure 5.1 *Mutagenesis of splice donor sites at exons 1_6, 1_{10} and 1_{11} in P2 does not decrease promoter activity.*
Transient transfection analysis in B103 (rat neuroblastoma) cells of a series of constructs in which the splice donor sites at exons 1_6, 1_{10} and 1_{11} were individually mutated within P2. The activity of all constructs is expressed relative to P2 nominally set at 100%. n=12-19 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to analysis by Student t-test.

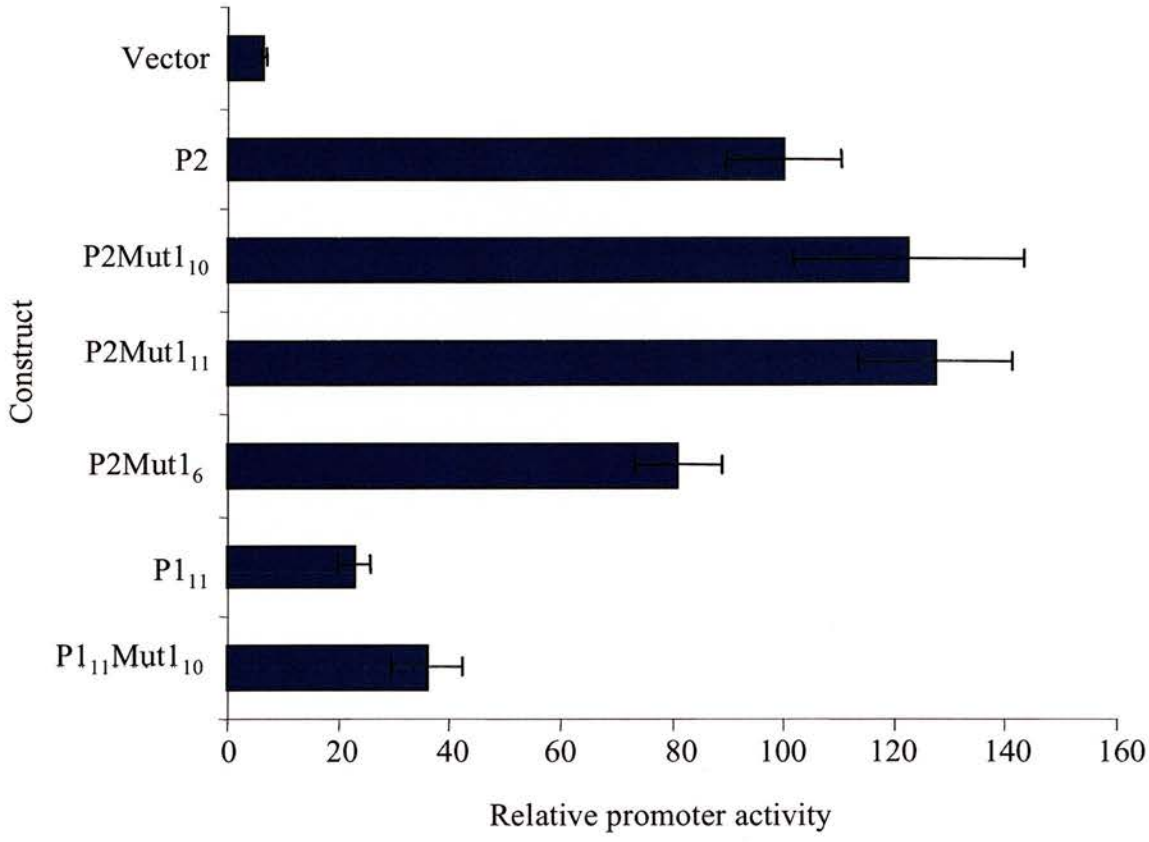
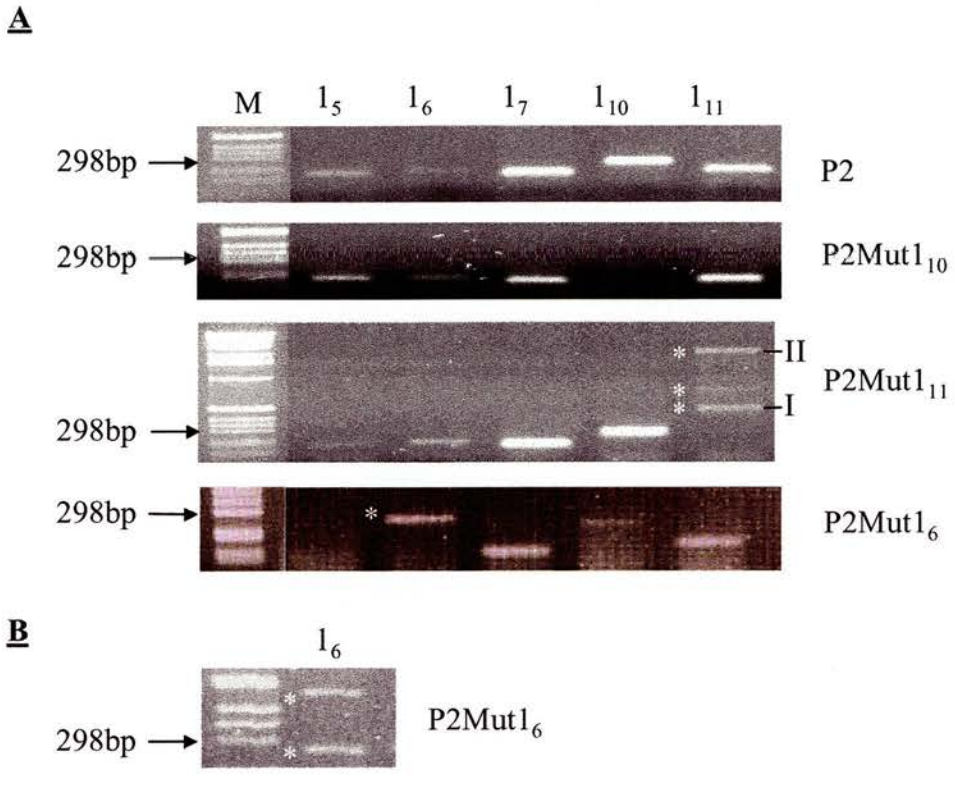

Vector
P2
P2Mut1$_{10}$
P2Mut1$_{11}$
P2Mut1$_{6}$
P1$_{11}$
P1$_{11}$Mut1$_{10}$
Construct
0
20
40
60
80
100
120
140
160
Relative promoter activity

Figure 5.2 *Longer transcripts are associated with GR exons 1 at which the splice donor sites are mutated.*
Representative gels showing RT-PCR analysis of RNA extracted from transfected B103 cells. Positive control reactions were carried out on RNA extracted from cells transfected with intact P2. Negative control reactions were also carried out for each primer pair using RT reactions minus AMV reverse transcriptase. No bands were observed in the negative controls (data not shown). '*' marks products of a larger size than predicted. 1kb ladder was run in lane M, and the 298bp band is indicated.
A: Results from RNA of B103 cells transfected with constructs including P2 and variants of P2 in which the splice donor sites at exons 1_6, 1_{10} and 1_{11} are mutated. Reactions used 5' primers specific to individual exons 1 (1_5, 1_6, 1_7, 1_{10} and 1_{11}) in conjunction with a common 3' primer specific to luciferase. 'I' and II' mark products detected in the exon 1_{11} reaction using RNA from P2Mut1_{11} transfected cells.
B: Further results from RNA of B103 cells transfected with P2Mut1_6.


A
M
1 5
1 6
1 7
1 10
1 11
298bp
P2
298bp
P2Mut1 10
II
I
P2Mut1 11
298bp
298bp
P2Mut1 6
B
1 6
298bp
P2Mut1 6

into pGEM-Teasy and sequenced. Sequence analysis confirmed that these longer products are spliced from GT donor sites located downstream of the mutated site, at -3001 and –2815 (fig.5.3). All other exon 1 products were detected at their predicted sizes.

Similarly, at least three longer exon 1_{11} products were detected in RNA extracted from cells transfected with P2Mut1_{11}. The longest and shortest of these transcripts (labelled I and II on fig 5.2) were sub-cloned into pGEM-Teasy and sequenced. Product I is spliced from a downstream splice donor site at –990 (fig. 5.4), while II corresponds to a full-length, unspliced transcript spanning the exon 1_{11} and luciferase primer sites (data not shown).

5.3.3 A 3.6kb *HindIII* fragment encompassing the CpG island region shows general *DNaseI* sensitivity.

Analysis of chromatin conformation was investigated by *DNaseI* hypersensitive site mapping. Genomic DNA was purified from *DNaseI*-treated H4IIE nuclei and restricted with *HindIII* to generate a 3641bp fragment encompassing the GR CpG island region (fig. 5.5A). The 3641bp 'parent' band can be seen to disappear with increasing *DNaseI* concentration (fig. 5.5B). Interestingly, the disappearance of the parent band is not accompanied by the appearance of any 'daughter' bands, which would correspond to sites of *DNaseI* hypersensitivity. As such, there appear to be no nuclease hypersensitive sites within the 3641bp *HindIII* fragment, rather the whole region is generally sensitive to *DNaseI* digestion.

5.3.4 A 30.1kb *SalI* fragment of the GR promoter contains a ~3kb 'daughter' band.

Results described above showed that the 3641bp *HindIII* fragment of the GR promoter showed general sensitivity to *DNaseI* digestion. In order to determine the boundaries of this sensitive region, *DNaseI* hypersensitive site mapping was carried out on genomic DNA purified from *DNaseI*-treated H4IIE nuclei and restricted with

Figure 5.3 *Sequence of longer transcripts generated by the P2Mut1$_6$ mutation.* Sequence of the GR promoter showing exons 1_6, 1_7 and 1_8 and the downstream splice donor sites used in the event of a mutation at the 1_6 splice donor sites. Exon 1 sequences present in 5'RACE-PCR clones identified by McCormick *et al* are underlined and labelled (McCormick et al., 2000). The GT to AC mutation at the 1_6 splice donor site is marked in red. The downstream splice donor sites of products identified by RT-PCR of RNA from B103 cells transfected with P2Mut1_6 are shown in blue and marked with triangles.

-3339 GGGCCGCCCAGACGCTGCGGGGGTGGGGGACCTGGCGGCAC

GCGAGTCCCCCCCCGGGCTCACAACATGTATGCGCTGACCCT

-3247 CTCCTCTGCGCTCCCCTCCCCAGGCCTCCCCAGAGGGCGTGT

CTGCAGTCCTGCCCCGAGAGCAAGCGGCCAGGGCTCTGCGG

-3164 CACCGTTTCCGTGCCATCCTGTAGCCCCTCTGCTAGTGTGAC

ACACTTCGCGCAACTCCGCAGTTGGCGGGCGCGGACCACCCC

-3080 TGCGGCTCTGCCGGCTGGCTGTCACCCTCGGGGGCTCTGGCT

GCCGACCCACGGGGCGGGCTCCCGAGCGGTTCCAAGCCTCG

-2997 GAGCTGGGCGGGGGCGGGAGGGAGCCTGGGAGAAGAGAAA

CTAAAGAAACTCGGTTTCCCTCCCAGGCCAGGTCGGCACCCG

-2915 CTGCCGCACTTTTTCTCGTTCCTTGGGTGGGGAAAGGCGAAG

CCGCGCGCCCCAGCGAGGCGATGCCCTGAGCCGCGGGCTTG

-2832 CAGGCGCCGTCGGGGCCGGGCTGGCGGGTACCGCGCGCTGG

GAGAAAAGAGGGCGAGGGCCACGGGCGCCCTTGCAGTTGCC

-2750 GACAGTCGCCAACAGGTTGCACGTTCCCCGCGGCCGCGCGGC

CCCTCGGGCGGGGAGCGGCCGGGGGTGGAGTGGGAGCGCGT

-2666 GTGTGCGAGTGTGTGCGC

Figure 5.4 *Sequence of longer transcripts generated by the P2Mut1$_{11}$ mutation.* Sequence of the GR promoter showing exon 1_{11} and the downstream splice donor site used in the event of a mutation at the 1_{11} splice donor site. The exon 1_{11} sequence present in 5'RACE-PCR clones identified by McCormick *et al* is underlined and labelled (McCormick et al., 2000). The GT to AC mutation at the 1_{11} splice donor site is marked in red. The downstream splice donor site used to generate a product identified by RT-PCR of RNA from B103 cells transfected with P2Mut1_{11} is shown in blue and marked with a triangle. A further product corresponded to the full, unspliced sequence between exon 1_{11} and exon 2 (data not shown).

-1817 GCGCGGCTCCGGGCTGCGGGCTTGTAGGGTGGATTGGG

GCTCACAGCCTGCAGCCCAGACTTCGCCCGCCCGGCCT 1_{11}

TATCTGCTAGAAGTGGGCGTGCCGCAGAGAACTCAACA

-1703 GACCTGGACACATTTCTCCCTTCACCTCCCACCTTTCTC

CCTCCTTCTCCCCCAACCCCACCCCCGACAACTTGGGCG

CTAGCTTTGGGGCATGATTTCGCGCCTGACTTTTCTGAG

-1586 TGGTCCCCTTTTAGAAAGAGACCCTCCCTAGCCGCAGT

TTTTGATGCAGCGATTTTTTTTTTTTTTTTGACAAGTGC

TACTTTGACATTTGAGGTTGCAGCCTCGGTAATTGCAGC

-1469 CTTACCACTTAAGACCCTGGGCAAGGTTCGTGTGACTA

ATGTCACAGGGTTATTTACAGTTTTAACTGGGGGATAA

ATGTCGCTTAAGGGAGCATCTTGTTTTATGAAGTGTTAC

-1354 GGTTTCGGGCTGGAAGGGGCAGTTGTCAAAAAAGCAG

GTCTGAAAATTCTTTAAGGTCTATTAGATATCTTACATT

TAGAGATCCTTATCAAAGGCATAGGACCGACCGGGGTT

-1240 CTGAGAGAGAAGCCCTTTACAGGGAAGAATCCTAGGGT

AGGTTCCACCCCTCTCCACCTTCCCTGAATTTCCCTTTC

AGAGAAGGTGGTCATACTTAATGTCTTGGTACAGGAAA

-1125 AGTTTACCATTGTATTGGGGATCCCAAATATATTTGTCA

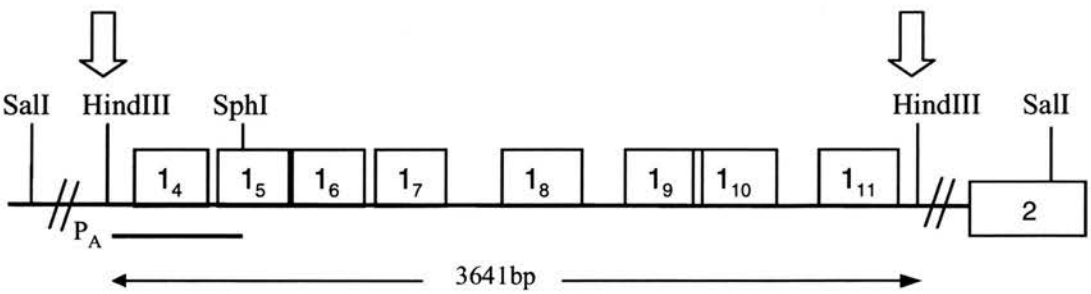
TAGTCTTTGCCAGCCCCTCAAAACATTTTGATTATTACT

AACATACTAGCAATCTGGAGGAATACAGTAAAGGTTTA

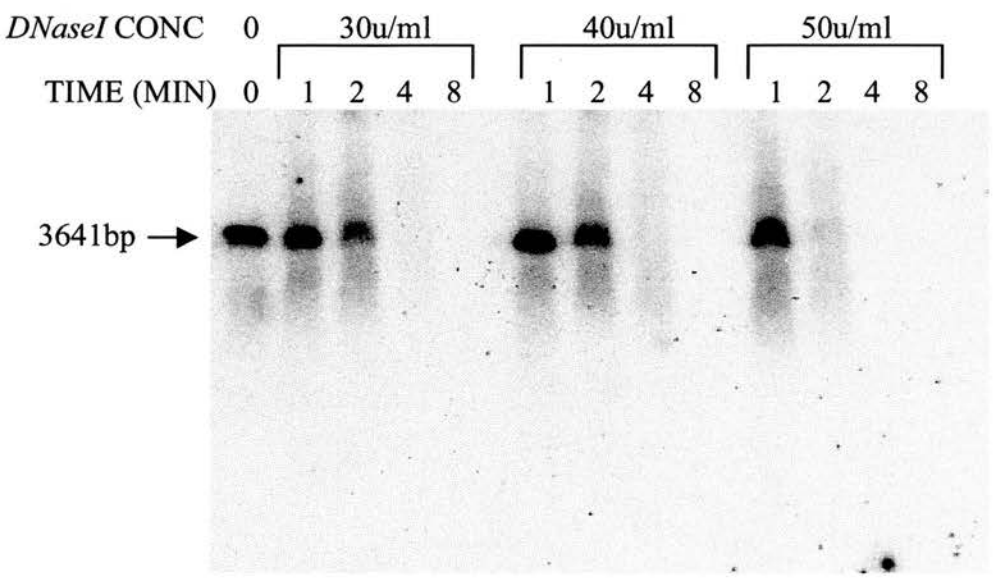
-1029 AAACTACAGAGAGTATTTTTTCTGAGCGTTTT

Figure 5.5 *A 3.6kb HindIII fragment encompassing the CpG island shows general DNaseI sensitivity.*
Strategy (A) and results (B) of *DNaseI* hypersensitive site mapping carried out on H4IIE nuclei. Nuclei were treated with 30U/ml, 40U/ml and 50U/ml *DNaseI* for 0, 1, 2, 4 and 8 min. Genomic DNA was purified before *HindIII*-restriction, Southern blotting and hybridisation to a *SphI/HindIII* radiolabelled probe (labelled P_A). The 3641bp parent *HindIII* fragment is indicated.

A


SalI
HindIII
SphI
HindIII
SalI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
P A
3641bp

B


DNaseI CONC 0 30u/ml 40u/ml 50u/ml
TIME (MIN) 0 1 2 4 8 1 2 4 8 1 2 4 8
3641bp

SalI. This digestion generated a 30.1kb fragment that extended from the beginning of exon 2 to close to exon 1_1 and therefore encompassed the whole *HindIII* region (fig. 5.6A). A 30.1kb parent band can be seen in the 'zero' *DNaseI* reaction but is absent from all *DNaseI*-treated reactions (fig. 5.6B). A 'daughter' band of ~3kb can be seen in three *DNaseI*-treated lanes (2, 6, 10), and is particularly strong in lane 10. Two further diffuse bands, one larger and one smaller than 3kb can also be seen in lanes 2,6 and 10.

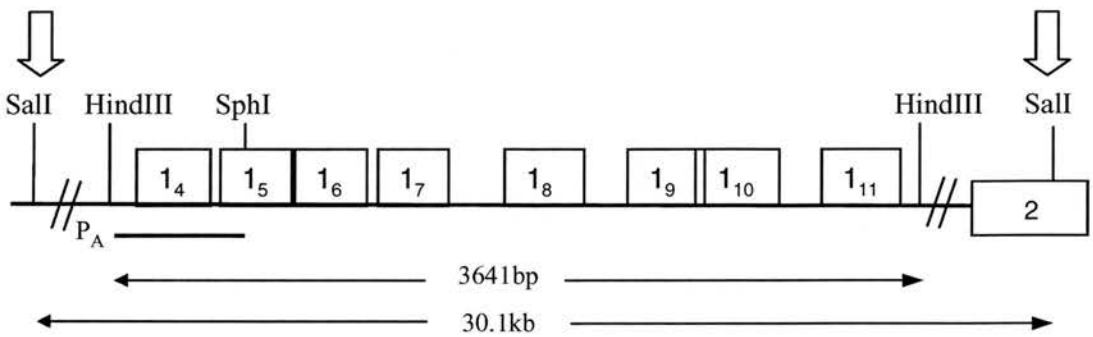
5.4 Discussion

The experiments described in this chapter were designed to investigate the role of alternate exons 1 in the constitutive regulation of GR. It was hypothesised that relatively indiscriminate transcription initiation over the whole CpG island combines to generate high GR expression. As such, the role of exon 1-associated splice donor sites was investigated. Specifically, the proposal that, following initiation, transcripts simply splice at the most proximal donor site was tested. This hypothesis also implies that a large region of transcriptionally active DNA extends over the GR promoter. This region should therefore be accompanied by a large area of 'open' chromatin; a proposal that was tested by *DNaseI* hypersensitive site mapping.

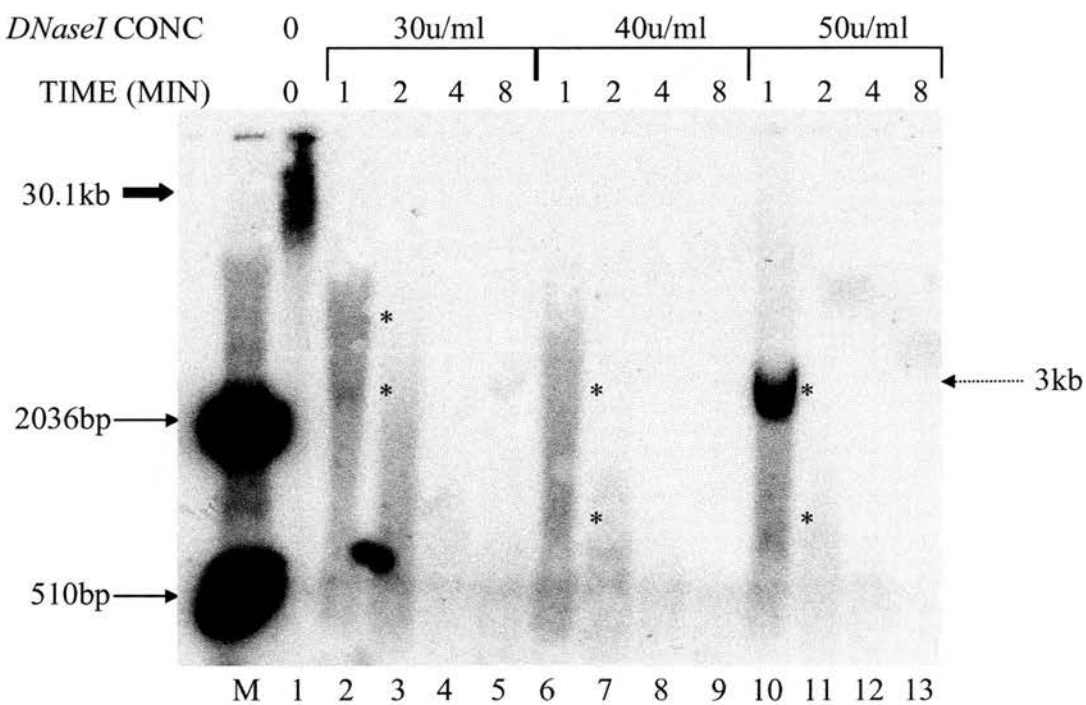
RT-PCR analysis of RNA extracted from B103 cells transfected with P2 confirmed that all exons 1 examined were present at their predicted sizes. This confirmed that all exon 1 transcripts identified *in vivo* can be detected in this *in vitro* system. The identification of longer products associated with mutations at the splice donor sites of exons 1_6 and 1_{11} also gives rise to several interesting implications. It appears that *RNA polymerase II* and its associated splicing machinery by-passes the mutation and continues to a donor site further downstream. Following sequence analysis showing that all splicing conformed to the GT/AC rule (Black, 2003), it was originally assumed that, after moving past the mutation, the splicing machinery would simply utilise the next suitable donor site. However, results from experiments in which the 1_6 site was mutated show that this was not always the case. Whilst the splicing machinery generated products that were spliced at –3001 and –2815, no product was

Figure 5.6 *A 30.1kb SalI fragment of the GR promoter contains a ~3kb 'daughter' band.*
Strategy (A) and results (B) of *DNaseI* hypersensitive site mapping carried out on H4IIE nuclei. Nuclei were treated with 30U/ml, 40U/ml and 50U/ml *DNaseI* for 0, 1, 2, 4 and 8 min. Genomic DNA was isolated before *SalI*-restriction, Southern blotting and hybridisation to a *SphI/HindIII* radiolabelled probe (P_A). 1kb ladder was run in lane M and the 2036bp and 510bp bands are indicated. '*' marks bands generated by cleavage at potential *DNaseI* hypersensitive sites. A solid arrow marks the 30.1kb parent fragment and a broken arrow indicates a ~3kb daughter band.

A


SalI
HindIII
SphI
HindIII
SalI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
P A
3641bp
30.1kb

B


DNaseI CONC
0
30u/ml
40u/ml
50u/ml
TIME (MIN)
0 1 2 4 8 1 2 4 8 1 2 4 8
30.1kb
2036bp
510bp
3kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13

generated from splicing at –2916, the mapped 1_7 donor site. It is unclear why this splice donor site, which generates the 1_7 transcript identified in both tissues and cell lines, is not utilised. However, it may be that the product generated by splicing at the 1_7 donor site is, in this case, inherently unstable and therefore rapidly degraded.

The absence of a longer exon 1_{10} transcript in RNA extracted cells transfected with P2Mut1_{10}, is also puzzling. It may be that the mutant 1_{10} product(s) is too large to amplify successfully under the PCR conditions used. However, use of the exon 1_{11} splice donor site in this instance would generate a product of 677bp, considerably smaller than the 800bp and 1650bp products successfully detected as a result of the 1_{11} mutation. Once again, it is possible that the longer transcripts may be inherently unstable and therefore rapidly degraded.

The splice donor sites identified during the experiments described above have never been detected in previous RT-PCR or 5'-RACE-PCR analyses in either cells or tissues. Initial 5'RACE-PCR data identified clones containing overlapping exons, e.g. a clone termed $1_{4.5}$ was spliced from a donor site located between those mapped for exon 1_4 and 1_5 (McCormick, 2000), but it would appear that the vast majority of splicing occurs at the mapped donor sites. It is interesting that, following mutation of one such 'consensus' site, splicing occurs at more than one alternative. The 'cryptic' splice donor sites uncovered by the mutations are therefore considerably 'weaker'. Sequence analysis of the mapped 'consensus' sites at exons 1_4, 1_5, 1_6, 1_7, 1_9, 1_{10} and 1_{11} revealed no obvious splicing enhancer elements (Dominski & Kole, 1994; Selvakumar & Helfman, 1999; Tian & Kole, 2001), although it is interesting to note that five of the sites contained the sequence $^{C}/_{G}$AGGT, while two contained AGT. In contrast, the sequences of the three 'cryptic' donor sites identified were more heterogeneous.

Results also showed that mutation of exon 1 splice donor sites, including those at the predominant exons 1_6 and 1_{10}, had no effect on overall promoter activity. This finding provides further intriguing implications. The splice donor site mutations give rise to transcripts with significantly longer 5' leader sequences, which might

conceivably adopt different secondary structures, yet high luciferase activity is maintained. The influence exerted by 5' leader sequences and their associated secondary structure on mRNA stability and translational efficiency is well documented (Kozak, 1991; Preiss & Hentze, 1999; van der Velden & Thomas, 1999). However, these data suggest that the length of the GR 5' leader sequence has no functional influence on protein expression. This is admittedly an artificial system in which luciferase, rather than GR protein, is synthesised, yet if a primary function of these alternate exons 1 is to influence GR protein levels through differential mRNA stability, localisation or translation, we might expect to observe an effect.

DNaseI hypersensitive site mapping was carried out to determine if the entire exon 1 region occupies an area of transcriptionally active and therefore 'open' chromatin. Results indicated that a 3.6kb *HindIII* fragment, encompassing the whole CpG island region, is sensitive to digestion by *DNaseI*, with no accompanying sites of hypersensitivity. The lack of hypersensitive (HS) sites in a region known to be highly transcriptionally active was surprising. However, it should be noted that chromatin can exist in several different states (Kornberg, 1977). The extremes of chromatin conformation are exemplified by the inert nucleosomal chromatin associated with bulk transcriptionally inactive DNA and the nuclease HS sites that occur at promoters from which the nucleosomes have been displaced (Tazi & Bird, 1990; Cuadrado et al., 2001). However, between the extremes there is an intermediate level of chromatin structure that is associated with transcribed sequences (Tazi & Bird, 1990). Chromatin of this kind is defined by moderately enhanced *DNaseI* sensitivity of its constituent DNA, usually over an extended region (Weintraub & Groudine, 1976). CpG islands are known to involve modification of nucleosomal histones (in particular, histone acetylation), absence of histone H1, presence of non-histone proteins and under-methylation of the DNA sequence (Tazi & Bird, 1990). These factors act to prevent higher order folding of chromatin at CpG islands, leading to a disorganised 'beads-on-a-string' nucleosomal structure. This kind of structure, typical of a CpG island, may be responsible for the long-range *DNaseI* sensitivity observed over the GR promoter. However, it must be stressed that the structural 'openness' of chromatin is a relative issue and all chromatin is *DNaseI* sensitive to

some degree. The *DNAseI* sensitivity of the *HindIII* fragment of GR can only be expressed relative to a control fragment from another gene. This control must be carried out in order to confirm the sensitivity of this region.

Further *DNaseI* hypersensitive site mapping was carried out to elucidate the boundaries of this sensitive region. Experiments showed that a large 30.1kb *SalI* 'parent' fragment extending from exon 1_1 to exon 2 yielded a ~3kb 'daughter' band following *DNaseI* treatment. Increased *DNaseI* exposure subsequently caused this ~3kb band to disappear. The probe used to detect this daughter band was located >25.2kb and 4.5kb away from the 5' and 3' *SalI* sites respectively. As such, both ends of the ~3kb fragment must be generated by *DNaseI*. It is extremely likely that this ~3kb fragment corresponds to the entire 3.6kb *HindIII* fragment shown previously to exhibit general nuclease sensitivity. In this way, hypersensitive sites marking the ends of the ~3kb region were identified by low concentrations of *DNaseI* (~50U/ml). This region, which must overlap with (and probably corresponds to) the 3.6kb *HindIII* fragment, shows general *DNaseI* sensitivity and is therefore degraded following increased nuclease exposure (>60U/ml). It is possible that both hypersensitive sites lie outside the 3.6kb *HindIII* fragment, hence the absence of a 'daughter' band in experiments using the 3.6kb *HindIII* 'parent' fragment. Similarly, if a hypersensitive site is present, its location must be extremely close to one of the *HindIII* restriction sites. The resulting 'daughter' band would either be too small to allow successful identification or too similar in size to the parent band to be sufficiently distinguished.

The observation of the 3kb DNaseI sensitive band is the product of a single experiment and must therefore be viewed with caution. It is possible that the band is a product of exogenous enzyme activity and further experiments are therefore needed to verify its existence. Unfortunately, it was extremely difficult to optimise the *DNaseI* hypersensitive site mapping technique. The CpG island region of the GR promoter is obviously extremely GC rich, which can lead to problems during the denaturation and hybridisation steps of the Southern blotting protocol. GC-rich DNA regions are often described as 'sticky', and can therefore cause high levels of non-

specific binding during hybridisation with a radiolabelled probe. Unfortunately, very high levels of 'background' hybridisation, due to non-specific binding, rendered many blots uninformative. In addition, on several occasions hybridised blots were completely blank, which may have been due to incomplete denaturation of this 'sticky' GC-rich region.

The original intention of the *DNaseI* hypersensitive site mapping experiments was to compare the chromatin conformation of the GR locus in different cell types, specifically the B103 (rat neuroblastoma) and the H4IIE (rat hepatoma) cell lines. However, results discussed in Chapter 4 show that B103 cells do not endogenously express GR and were therefore unsuitable for this analysis. Time constraints imposed by the technical difficulties described above meant further experiments in alternative cell lines could not be carried out. However, elucidation of the chromatin structure at the GR locus in different cell types is an important part of investigations into GR regulation, and should be pursued. In particular, comparison of the non-CpG island exon 1_1 region between expressing and non-expressing cells would be extremely interesting. Previous studies have shown sites of *DNaseI* hypersensitivity at the 1A promoter region in WEHI-7 T-lymphoma, but not LTK$^-$, cells (Strahle et al., 1992). We might therefore speculate that the CpG island region, which is ubiquitously active, is in 'open' chromatin conformation in all cell types, while a tissue-specific hypersensitive site at exon 1_1 restricts its expression to T cells only.

The data presented here indicates that splice donor site selection has no impact on overall GR promoter activity, as well as demonstrating that the CpG island region occupies an extended area of transcriptionally active chromatin. These findings are consistent with the hypothesis of relatively indiscriminate transcription initiation over the whole region, which combines to generate high overall expression. However, this raises the question of why expression is generated by the combined activity of several 'weak' promoters, rather than one 'strong' promoter. There are many genes whose 'housekeeping' roles require their guaranteed expression in every cell type at all times, regardless of the typical pool of transcription factors present. It is conceivable that one single promoter may simply not be able to accommodate all

the necessary elements for truly ubiquitous expression. This hypothesis is supported by work carried out on the housekeeping gene Hft9 showing that its promoter binds different factors depending on the tissue in which it is expressed. As such, cell type-specific combinations of tissue-specific and ubiquitous binding factors act to guarantee Hft9 expression in every cell (Stapleton et al., 1993).

There is also the intriguing possibility that a gene in which transcription is initiated at several sites, generating transcripts differing only in their 5'-leader sequence, might be rendered less vulnerable to mutations in promoter sites. This would obviously be of enormous benefit to a housekeeping gene whose expression must be guaranteed in every cell. In this light, heterogeneity of transcription initiation at the GR CpG island might simply be a robust mechanism to ensure ubiquitous expression. Furthermore, CpG islands are generally believed to be under a greater mutational pressure relative to the rest of the genome because of their dual role as promoters and DNA replication origins (Delgado et al., 1998; Antequera & Bird, 1999; Cuadrado et al., 2001). Maintaining ubiquitous GR expression in the face of this large mutational burden may therefore require the protection of multiple redundant promoters.

There are several reported cases of genes containing both a promoter that confers ubiquitous expression and an alternative promoter with a more restricted spatial or temporal expression pattern. As mentioned in section 1.2.2.2.3.2, the human phobilinogen deaminase (PGBD) gene exemplifies this situation, displaying a CpG island-embedded promoter that is active in all tissues and an alternative promoter, located 3kb downstream, which is active only in erythroid cells (Chretien et al., 1988). The human STAT5B gene, which belongs to the Signal Transducer and Activators of Transcription family of transcription factors, employs a similar mechanism in which constitutive and tissue-specific activity is driven by promoters located in and out of a CpG island respectively (Ambrosio et al., 2002). It is not difficult to apply this system to the regulation of GR, in which constitutive expression is driven by multiple promoters in the CpG island region, while the upstream exon 1_1 promoter is tissue-specific.

The results described in this chapter are consistent with the hypothesis that the CpG island multiple exon 1 structure plays a major role in the constitutive expression of GR. As such, an extended region of 'open' chromatin facilitates heterogeneous transcription initiation over the whole CpG island region. The presence of multiple transcription initiation sites allows the GR promoter to exhibit a great deal of resistance to mutation, even if the disruption is not 'random' but directed at the splice donor sites crucial in maintaining 'normal' exon 1 structure. The resulting multiple exon 1 structure of the GR promoter ensures robust, high throughput and constitutive expression of this essential housekeeping gene.

6 Investigation of the role of the GR promoter and its constituent alternate exons 1 in rat GR autoregulation.

6.1 Introduction & Aims

Work described in the previous three chapters investigated the complex rat GR promoter and its associations with tissue-specific and constitutive GR regulation. However, an investigation into GR regulation must also consider the most potent regulators of GR expression: glucocorticoids themselves. There is a wealth of evidence showing that regulation of GR by glucocorticoids is a GR-mediated response, with repression predominating in most cells and tissues (section 1.2.2.2.1)

The experiments described in this chapter were designed to investigate the effect of the synthetic glucocorticoid, dexamethasone, on the rat GR promoter. In particular, the hypothesis that individual GR exons 1, or discrete elements of the promoter, are differentially regulated by dexamethasone was investigated. Previous work carried out *in vivo* has suggested that glucocorticoid-induced repression of GR reaches a 'ceiling', after which increased glucocorticoid exposure causes no further decrease in GR expression (Sapolsky et al., 1984). In this way, it was hypothesised that specific exons 1 may be targets for autoregulation, while others act to maintain GR expression, even at high glucocorticoid levels.

6.2 Experimental Design

To investigate potential autoregulation of the GR promoter, the P2 construct was transiently transfected into B103 cells that were then treated with either vehicle or the synthetic glucocorticoid dexamethasone. Results described in chapter 3 showed that B103 cells do not endogenously express GR. Two different masses of the pRShGR expression plasmid, 10ng and 100ng, were therefore co-transfected in all transfections. One hour post-transfection, cells were treated with vehicle (0.1% ethanol), 10^{-8}M or 10^{-6}M dexamethasone. The higher dose has frequently been used in both *in vivo* and *in vitro* experiments, while the lower dose is closer to the Kd of

dexamethasone for GR and is therefore more physiologically relevant. Unlike previous transfection experiments, cell medium was not changed 24h post-transfection and cells remained in the 'transfection' medium until harvesting.

6.3 Results

6.3.1. P2 activity is decreased following 48h dexamethasone treatment.

Results showed a significant decrease in P2 activity in all dexamethasone-treated transfections (fig. 6.1). In addition, experiments in B103 cells, which do not endogenously express GR, showed that dexamethasone had no effect on P2 activity in the absence of a co-transfected GR expression plasmid, pRShGR (data not shown). This confirms that autoregulation of the GR promoter is dependent upon GR itself. Using 10ng co-transfected pRShGR, there was a significant decrease in activity between 10^{-8}M and 10^{-6}M dexamethasone treatment. However, using 100ng co-transfected GR plasmid, there was no significant difference in activity between dexamethasone concentrations. Similarly, using 10^{-8}M dexamethasone, there was a significant decrease in activity with 100ng relative to 10ng co-transfected GR plasmid. Finally, using 10^{-6}M dexamethasone, there was no significant difference in activity between groups co-transfected with either 10ng or 100ng pRShGR.

The decrease in P2 activity following dexamethasone treatment confirmed the presence of elements in the promoter that may be subject to autoregulation. 100ng pRShGR and 10^{-6}M dexamethasone were shown to cause the greatest reduction in P2 activity, and were therefore chosen for further transient transfection experiments.

6.3.2 Transfections using a 5' deletion series of the GR promoter identified a region subject to down-regulation by dexamethasone.

The 5' deletion series of P2 was transiently transfected into B103 cells in order to localise the dexamethasone-sensitive region (fig. 6.2). No significant difference in activity was observed between vehicle and 10^{-6}M dexamethasone-treated empty

Figure 6.1 *P2 activity is decreased following 48h dexamethasone treatment.* Transient transfection analysis of P2 in B103 (rat neuroblastoma) cells. Vehicle-treated P2 activity was nominally set to 100% and the activity of all other groups is expressed relative to this value. n=7-10 (2 independent plasmid DNA preparations), means ±SEM. Values were subject to Student t-test analysis. '*' denotes a significant difference from vehicle-treated P2 activity ($p<0.05$). '†' denotes a significant difference between groups indicated by brackets ($p<0.001$).

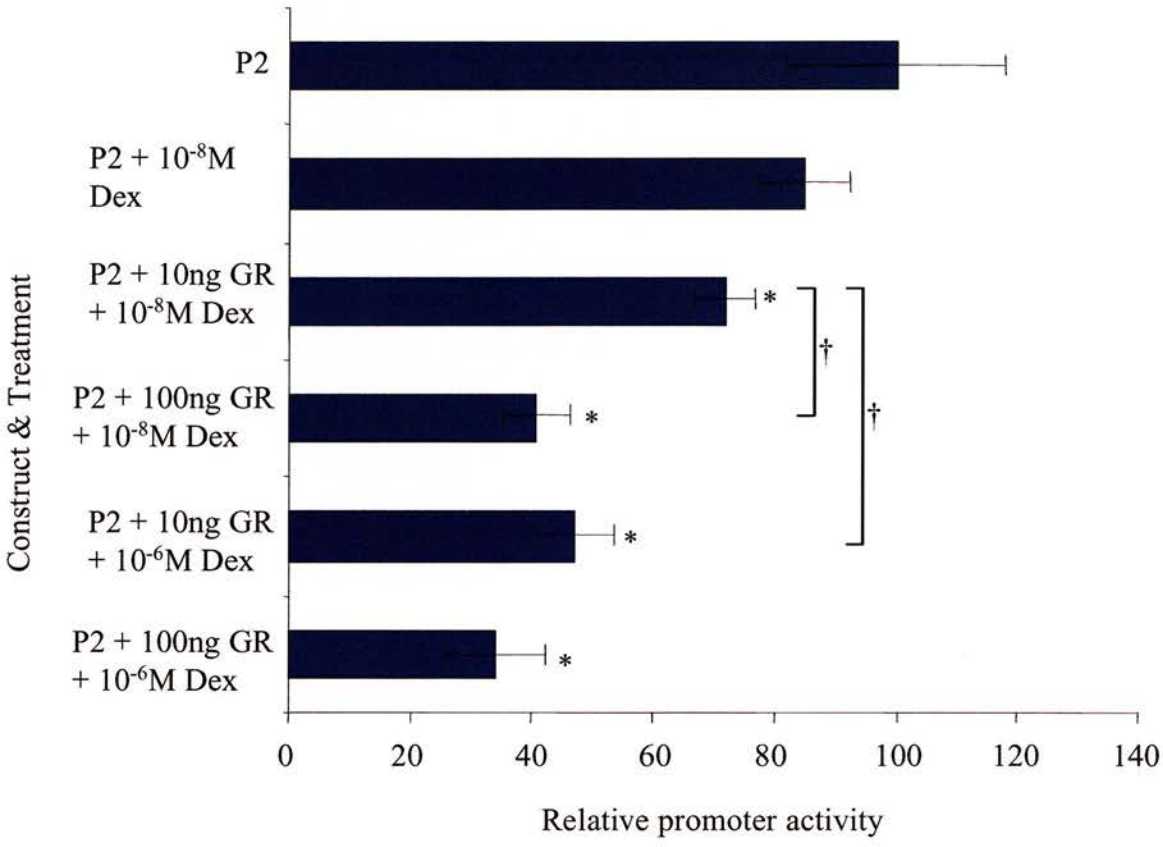
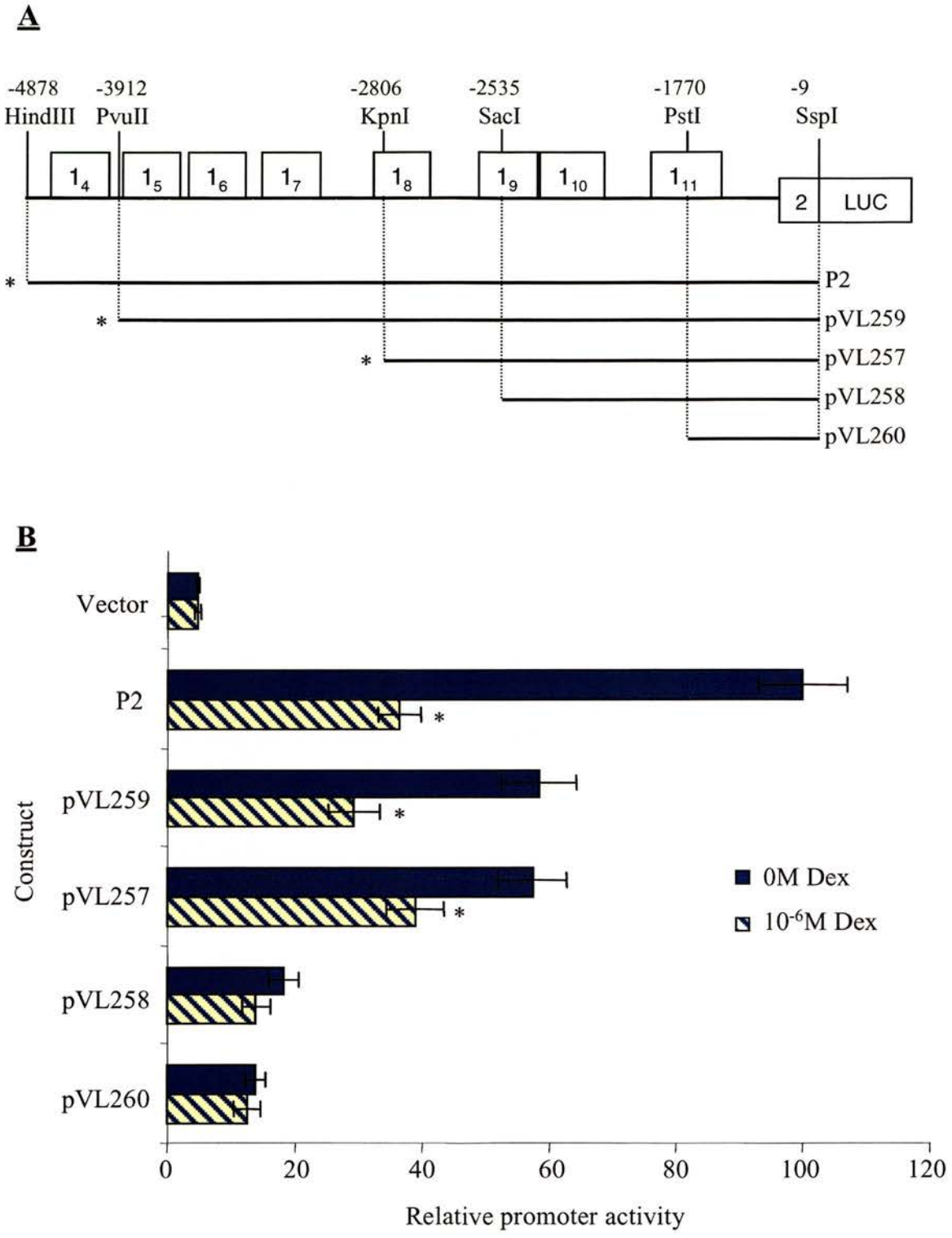

P2
P2 + 10-8M Dex
P2 + 10ng GR + 10-8M Dex
P2 + 100ng GR + 10-8M Dex
P2 + 10ng GR + 10-6M Dex
P2 + 100ng GR + 10-6M Dex
Construct & Treatment
0
20
40
60
80
100
120
140
Relative promoter activity
*
*
*
*
†
†

Figure 6.2 *Transfections using the 5' deletion series of the GR promoter identified a region associated with down-regulation by dexamethasone.*
A: Diagram showing the design of the 5' deletion series in which each construct is fused to a luciferase reporter gene. All constructs have the same 3' end but differ at the 5' end. The 3' end of each construct is fused to luciferase within exon 2. Restriction enzymes sites used to engineer constructs are also shown. '*' marks constructs showing a significant difference between dexamethasone-treated activity and vehicle-treated activity ($p<0.001$).
B: Transient transfections carried out in B103 (rat neuroblastoma) cells co-transfected with 100ng pRShGR and treated with either vehicle (0.1% ethanol) or 10^{-6}M dexamethasone. The activities of all constructs are expressed relative to a vehicle-treated P2 value nominally set at 100%. n=9-21 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to analysis by Student t-test. '*' denotes a significant difference between dexamethasone-treated activity and vehicle-treated activity for each construct ($p<0.001$).


A
-4878
HindIII
-3912
PvuII
-2806
KpnI
-2535
SacI
-1770
PstI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P2
pVL259
pVL257
pVL258
pVL260
B
Vector
P2
pVL259
pVL257
pVL258
pVL260
Construct
0M Dex
10-6M Dex
0
20
40
60
80
100
120
Relative promoter activity

vector (fig. 6.2B). All observed repression is therefore attributable to the cloned fragment. Dexamethasone treatment caused significant decreases in the activity of pVL259 (-3912 to -9) and pVL257 (-2806 to -9), but not pVL258 (-2535 to -9) or pVL260 (-1770 to -9). Constructs in which repression was observed therefore share a common dexamethasone-sensitive region between -2806 and –2535.

6.3.3 Dexamethasone significantly decreases the activity of all constructs in a 3' deletion series of the GR promoter.

Transient transfections of the 3' deletion series of P2 were undertaken to further localise the region of dexamethasone-sensitivity (fig. 6.3). Again, dexamethasone treatment had no significant effect on empty vector activity (fig. 6.3B). In contrast, P2 and all other constructs in the 3' deletion series showed a significant decrease in activity with dexamethasone treatment. As such, a region between –4878 and –3339, present in all constructs, appears to be dexamethasone-sensitive.

6.4 Discussion

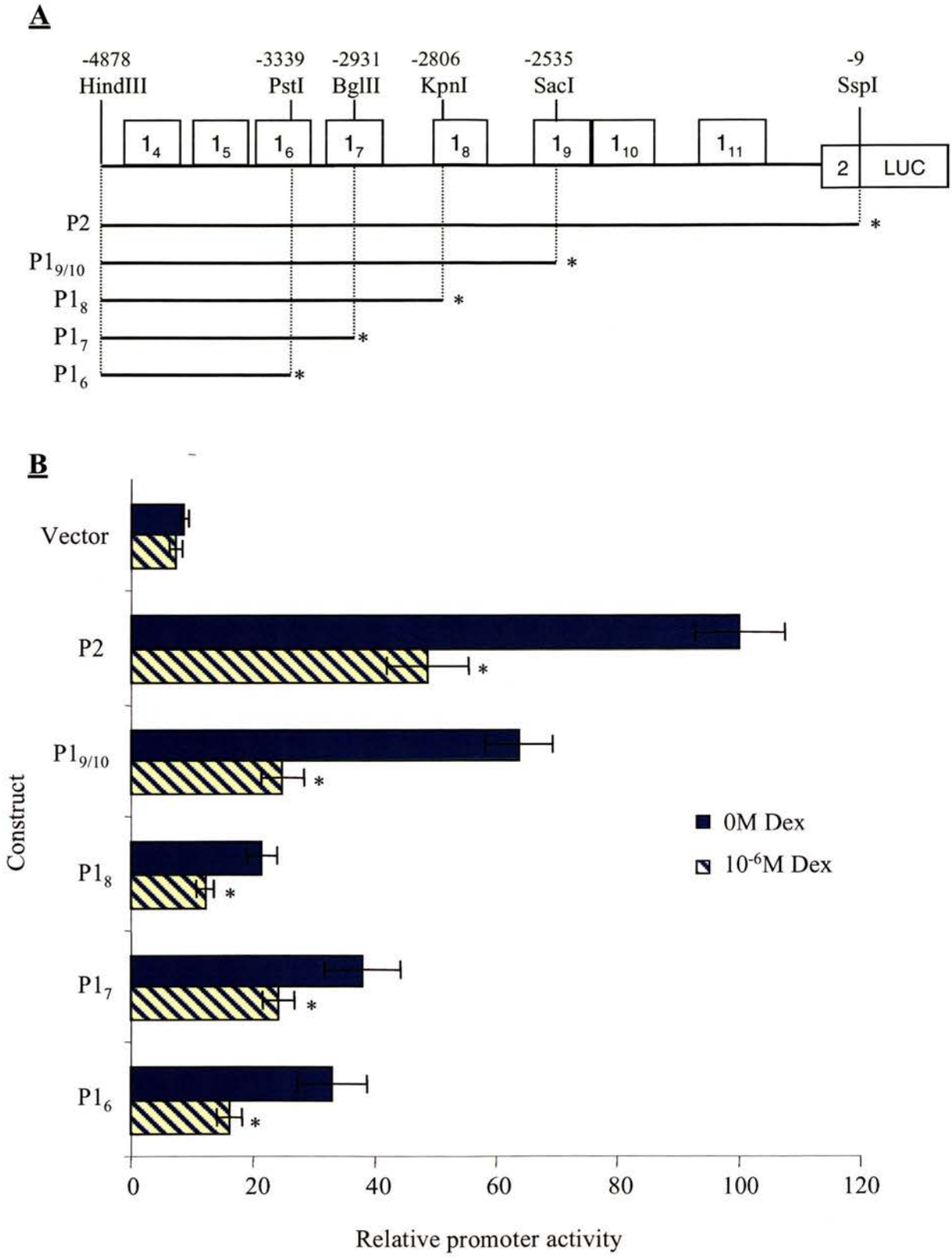
Experiments described in this chapter investigated the effects of the synthetic glucocorticoid dexamethasone on the rat GR promoter. Results also indicated that the extent of repression was dependent upon both the amount of co-transfected pRShGR expression plasmid and dexamethasone concentration. Interestingly, at the highest amount of pRShGR (100ng), there was no difference in P2 activity between 10^{-8}M and 10^{-6}M dexamethasone treatment groups. Similarly, at the highest concentration of dexamethasone (10^{-6}M), increased amounts of co-transfected pRShGR caused no further decrease in promoter activity. It appears, therefore, that both GR and glucocorticoid concentration are limiting in their effect on GR promoter activity.

Transient transfections using a 5' deletion series of P2 showed that dexamethasone-associated repression was lost following deletion of the region between –2806 to –2535. To investigate this further, the –2806 to –2535 region was cloned into a modified form of pGL3-Basic. The resulting construct (pHLM7) was then

Figure 6.3 *Dexamethasone significantly decreased the activity of all constructs in a 3' deletion series of the GR promoter.*

A: Diagram showing the design of the 3' deletion series, in which each construct is fused to a luciferase reporter gene. All constructs have the same 5' end but differ at the 3' end. The 3' end of P2 is fused to luciferase within exon 2 while all other constructs are fused within known exons 1. Restriction enzyme sites used to engineer the construct series are also indicated. '*' marks constructs showing a significant difference between dexamethasone-treated activity and vehicle-treated activity ($p<0.05$).

B: Transient transfections carried out in B103 (rat neuroblastoma) cells, co-transfected with 100ng pRShGR and treated with either vehicle (0.1% ethanol) or 10^{-6}M dexamethasone. The activities of all constructs are expressed relative to a vehicle-treated P2 value nominally set at 100%. n=11-12 (2 independent plasmid DNA preparations), values represent means ±SEM. Data were subject to analysis by Student t-test. '*' denotes a significant difference between dexamethasone-treated activity and vehicle-treated activity for each construct ($p<0.05$).


A
-4878
HindIII
-3339
PstI
-2931
BglII
-2806
KpnI
-2535
SacI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P2
P1 9/10
P1 8
P1 7
P1 6
B
Vector
P2
P1 9/10
P1 8
P1 7
P1 6
Construct
0M Dex
10-6M Dex
0
20
40
60
80
100
120
Relative promoter activity

transfected into B103 cells and subject to either vehicle or dexamethasone treatment. Unfortunately, in this set of transfections, dexamethasone failed to induce a significant decrease in P2 activity. There was therefore no positive control for dexamethasone efficacy. Several different batches of dexamethasone were tested but none caused the reduction in P2 activity seen in previous experiments. The reason for this failure remains elusive.

Results also demonstrated that all constructs tested in the 3' deletion series of P2 (specifically $P1_{9/10}$, $P1_8$, $P1_7$ and $P1_6$) were down-regulated by dexamethasone. These constructs share a common region between –4878 and –3339, suggesting that this area contains elements associated with dexamethasone sensitivity. However, transient transfections involving shorter constructs in the 3' deletion series, such as $P1_5$ and $P1_4$, are required to further localise this region. Together, data from the 5' and 3' suggest that two regions of P2, from –4878 to –3339 and from –2806 to –2535 are associated with dexamethasone repression (summarised in fig. 6.4).

However, it must be remembered that the luciferase fusion in each of the 3' deletion constructs abolishes the splice acceptor site normally present at the beginning of exon 2 (see section 4.2.1). As such, only the fused exon contributes to luciferase activity; all upstream transcripts are predicted to be spliced at their respective donor sites but, in the absence of a suitable acceptor site, are unlikely to give rise to functional luciferase activity (fig. 6.5A). The region between –4878 and –3339 that is common to all 3' deletion constructs must therefore exert a repressive effect on transcription from downstream initiation sites in response to dexamethasone (fig. 6.5B). In this way, the region can be thought of acting as a 'repressor'; factors binding in this region can be seen to have a negative effect on the activity of downstream promoters in a manner similar to the positive effect associated with 'enhancers'.

Previous work using constructs in which the human GR promoter was fused directly to a CAT reporter gene showed dexamethasone-associated repression when transfected into CV-1 cells (Govindan et al., 1991). The area of dexamethasone

Figure 6.4 *Two regions of P2, –4878 to –3339 and –2806 to –2535, appear to be dexamethasone-sensitive.*
Diagram showing the 5' and 3' deletion series used in transient transfection experiments to localise regions of dexamethasone-sensitivity. Common regions shared by constructs exhibiting dexamethasone repression are shown in red.

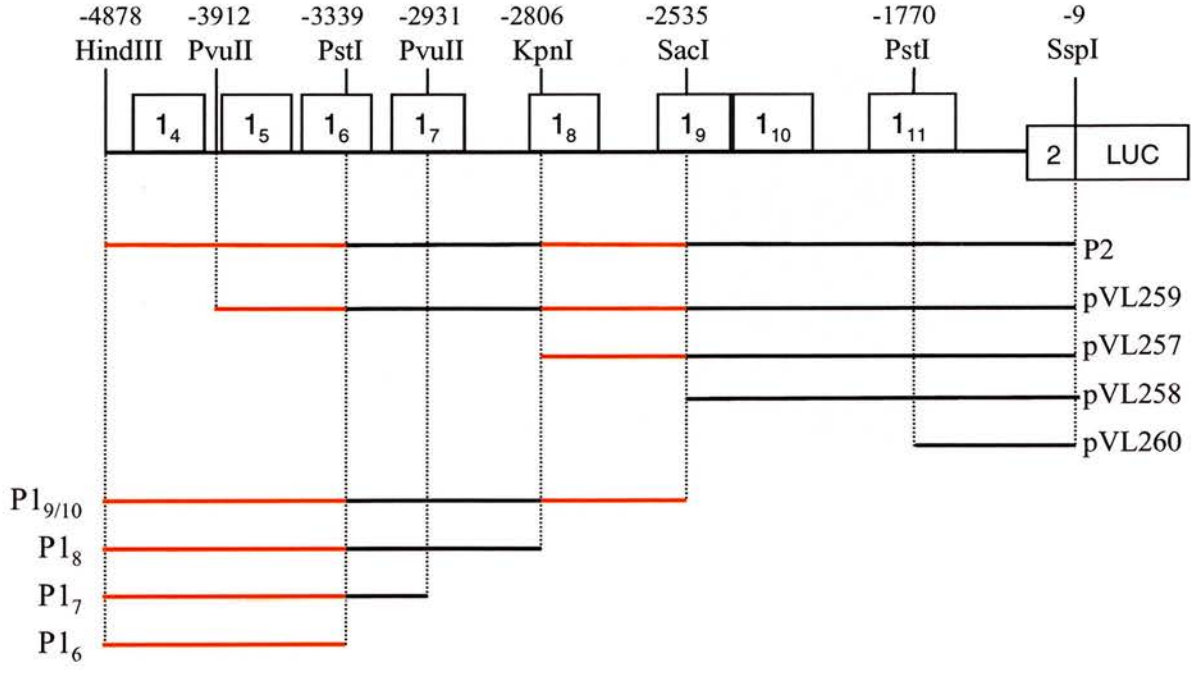
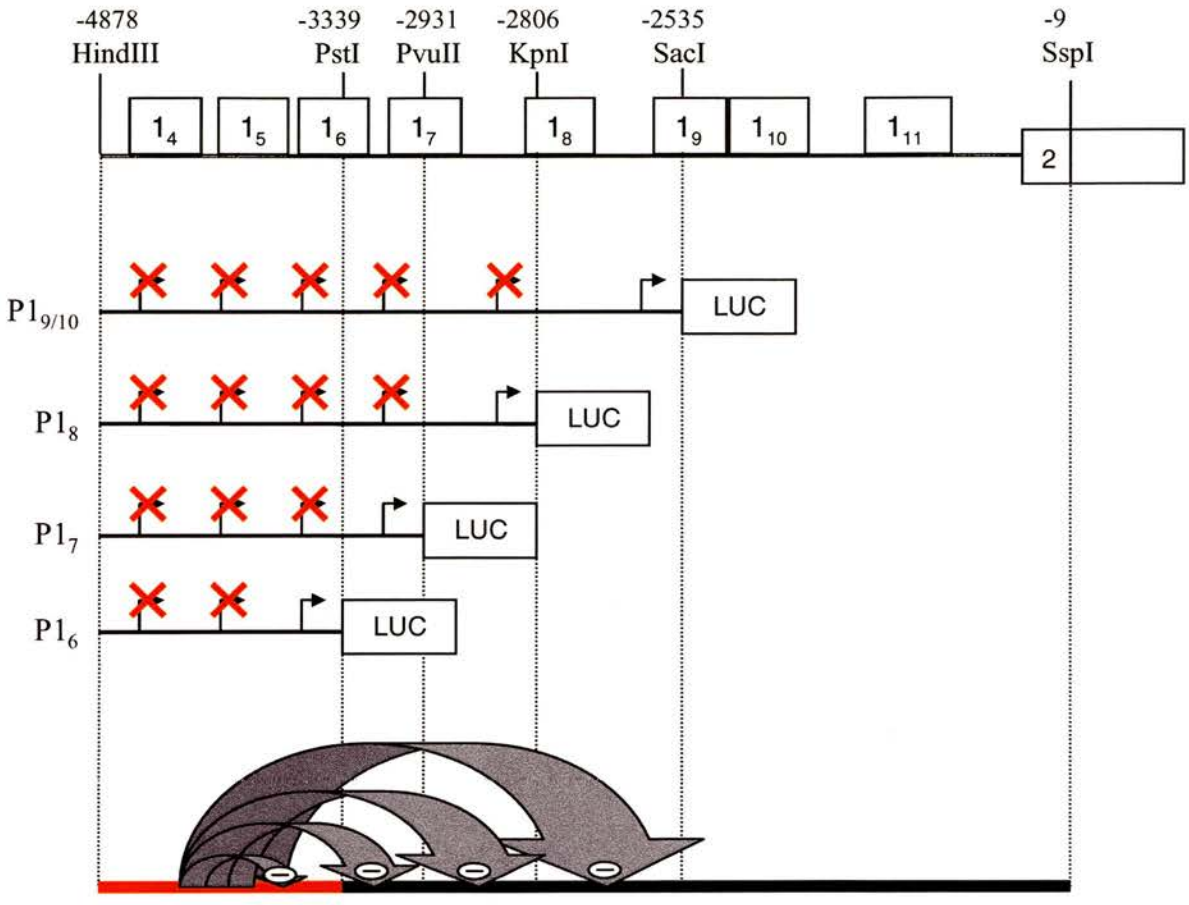

-4878
HindIII
-3912
PvuII
-3339
PstI
-2931
PvuII
-2806
KpnI
-2535
SacI
-1770
PstI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
LUC
P2
pVL259
pVL257
pVL258
pVL260
P1 9/10
P1 8
P1 7
P1 6

Figure 6.5 *The dexamethasone-sensitive region of P2 between –4878 and –3339 must exert downstream effects.*
A: Diagram showing the 3' deletion series used in transient transfection experiments to localise regions of dexamethasone sensitivity. Arrows indicate putative transcription initiation sites; those which do not give rise to functional luciferase activity are crossed out. 'LUC' represents the luciferase gene, fused to each 3' deletion construct within a known exon 1.
B: Diagram depicting the putative region of dexamethasone-sensitivity, with arrows representing its possible downstream effects.


-4878
HindIII
-3339
PstI
-2931
PvuII
-2806
KpnI
-2535
SacI
-9
SspI
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2
P1 9/10
P1 8
P1 7
P1 6
LUC
LUC
LUC
LUC

sensitivity was mapped between –750 and –250 relative to the transcription start at exon 1_{10} (Govindan et al., 1991) and therefore corresponds roughly to the region between –3156 and –2656 on the rat GR promoter. This region overlaps with the region of dexamethasone sensitivity between –2806 and –2535 localised during this investigation. Interestingly, a novel protein present in MCF-7 cell extracts, termed GRF-1, was shown to bind to this region (Leclerc et al., 1991a; Leclerc et al., 1991b). However, while GRF-1 mRNA was shown to be up-regulated in rat liver following dexamethasone treatment, this was not found to be the case in the MCF-7 cell line from which the protein was isolated (Warriar et al., 1996). In addition, this result is contradicted by several studies showing that homologous down-regulation of GR mRNA and protein appears to be independent of protein synthesis, since it occurs in the presence of cylcohexamide (Okret et al., 1986; Burnstein et al., 1994).

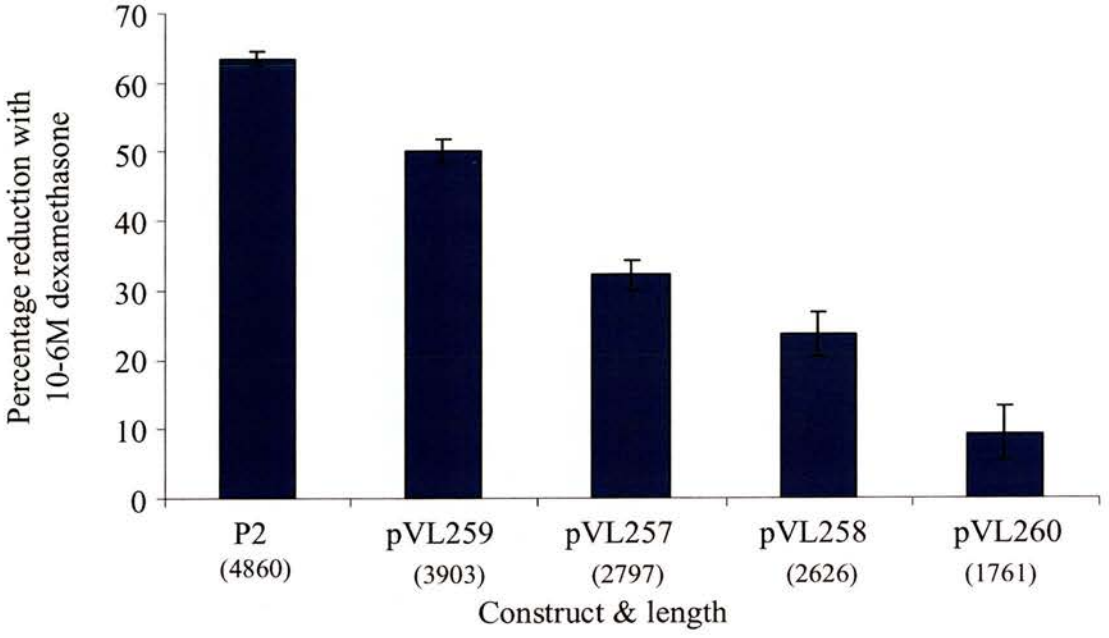
The latter observation implies that activated GR does not induce expression of a factor that subsequently represses GR transcription. Thus, the inhibitory actions of activated GR must occur through *direct* interactions of either the (i) protein-protein or (ii) protein-DNA variety. With regard to possibility (i), it may be that activated GR recruits a factor(s) that directly inhibits GR transcription, or binding by activated GR prevents a factor(s) from maintaining GR transcription. Investigation of possibility (ii) is hindered by the heterogeneous nature of negative GRE elements (see section 1.2.1.5). However, two sequences have been found in the human GR promoter, in the region between –2838 and –1476 (relative to the translation start) that are homologous to the nGREs found in the promoter of the POMC gene (Drouin et al., 1989; Schaaf & Cidlowski, 2003). While it remains to be seen if this region is involved in human GR autoregulation (Schaaf & Cidlowski, 2003), it is interesting that it roughly corresponds to the –2806 to –2535 dexamethasone sensitive region suggested by these experiments, although the exact sequences are not conserved. Further investigation to confirm interactions between GR and sequences in this region are required to confirm the presence of putative nGRE sequences. It should also be noted that sequence analysis of the –4878 to –3339 region, which was also associated with dexamethasone repression in these experiments, did not identify any similar nGRE elements.

However, further analysis of the transfection data suggested an alternative interpretation. Interestingly, the reduction in activity exerted by dexamethasone in the 5' series appears to be proportional to construct length (fig. 6.6A). That is, the longer the construct, the greater the repression. The exon 2 splice acceptor site lies upstream of the luciferase fusion in all constructs of this series. Thus, each construct reflects the total activity associated with all included exons. The deletion of exons therefore appears to be proportionally associated with a decrease in the extent of dexamethasone down-regulation. This interpretation suggests that the mechanism of autoregulation does not act at discrete sites on the promoter, but occurs in a manner dependent upon the number of alternate exons 1 available. In this way, repression of the two shortest constructs, pVL258 and pVL260, might simply be below the limit of detection of the transient transfection technique. The power of the transient transfection technique is enhanced by experiments in which the potential regulation associated with a region is 'transferred' or mutated. However, mutation is impossible without more exact knowledge of the location of any potential regulatory region and there would be little benefit in transferring the regions contained in pVL258 and pVL260 into an alternative promoterless vector. Nevertheless, while these experiments may not be sufficiently powered to reliably detect significance at these small levels of GR repression, changes of this magnitude may exert considerable physiological influence.

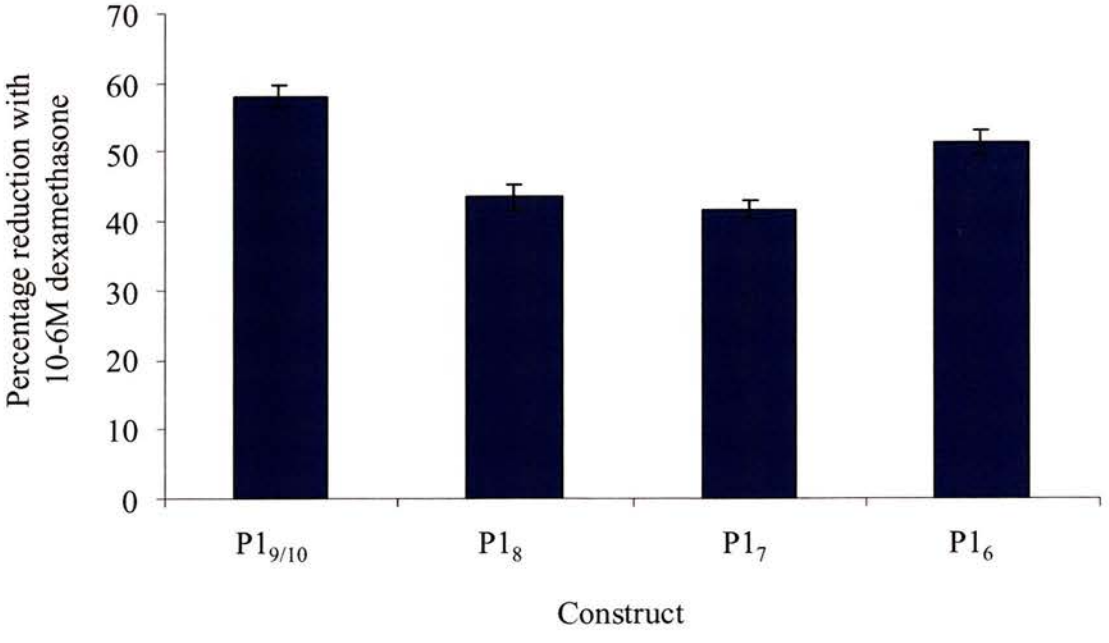
Results from the 3' deletion series transfection data also conform to this alternative interpretation. If, as discussed above, there are two dexamethasone-sensitive regions, constructs in which *both* are present should show significantly greater repression. However, repression of $P1_{9/10}$, which possesses both proposed dexamethasone-sensitive regions, is not significantly greater than that of $P1_8$, $P1_7$ or $P1_6$. Instead, dexamethasone exerts a similar repressive effect of ~50% on the activity of all constructs in the 3' deletion series (fig. 6.6B). The 3' deletion series measures the transcriptional activity associated with each individual exon 1, and as such, dexamethasone can be thought of as exerting the same effect, to the same extent, on the promoter activity associated with each exon 1. If the extent of autoregulation depends upon the number of alternate exons 1 available; all 3'

Figure 6.6 *Patterns of dexamethasone repression in the 5' and 3' deletion series of P2.*
Graphs showing dexamethasone repression of the 5'(A) and 3' (B) deletion series of P2. Values represent the percentage repression in promoter activity associated with dexamethasone. The value for P2 was generated from all experiments shown in figs. 6.1, 6.2 and 6.3 (n=36).

A. 5' Deletion Series



B. 3' Deletion Series



deletion constructs, in which only the fused exon contributes to activity, should, and do, exhibit the same extent of dexamethasone repression.

The mechanism that might cause dexamethasone repression to occur in a manner dependent upon the number of alternate exons 1 available is unclear. If correct, such a mechanism would be limited by either the number of transcription initiation sites available or the number of transcripts bearing different 5' leader sequences. The first possibility is unlikely because, while only members of the 5' series show variable repression, both deletion series possess a variable number of transcription initiation sites (albeit without the formation of viable transcripts in the 3' series). The second possibility evokes a post-transcriptional mechanism in which the presence of GR transcripts bearing a larger number of different 5' leader sequences somehow permits greater repression. However, it should be noted that speculation is limited by the fact that any post-transcriptional regulation is acting on luciferase, rather than GR mRNA. It must also be remembered that any putative mechanism of down-regulation must occur without, as previously mentioned, any *de novo* protein synthesis. As such, activated GR must act directly to effect any transcriptional or post-transcriptional changes.

Previous work has shown that autoregulation of GR can occur at a post-transcriptional level. While some groups have failed to observe any change in GR mRNA stability with glucocorticoids (Rosewicz et al., 1988; Dong et al., 1988), Burnstein *et al* described a greater than 2-fold increase in GR mRNA degradation following dexamethasone treatment (Burnstein et al., 1994). It would be interesting to investigate associations between multiple 5' leader sequences and GR mRNA stability after steroid treatment. Indeed, GR minigene constructs have been synthesised in this laboratory in order to address this question.

Transient transfection experiments using GR expression vectors under the control of either the Rous sarcoma virus (Burnstein et al., 1991) or human metallothionein-IIa (Alksnis et al., 1991) promoters, also showed a decrease in GR mRNA after steroid treatment, again suggesting a post-transcriptional mechanism of autoregulation.

However it should be noted that, in these instances, autoregulation occurs in the absence of any 5' leader sequences, although evidence does indicate the involvement of the 3' untranslated region (Okret et al., 1986).

Evidence indicates that autoregulation of GR operates at multiple levels, affecting both mRNA and protein. Transcription rate (Dong et al., 1988) (Rosewicz et al., 1988), mRNA stability (Burnstein et al., 1994), and protein degradation (McIntyre & Samuels, 1985) have all been implicated in mediating the glucocorticoid response. Given that autoregulation of GR is influenced by factors including tissue-specificity (Kalinyak et al., 1987), stage of cell cycle (Cidlowski & Cidlowski, 1982), degree of cellular differentiation and aging, perhaps the existence of multiple levels of control is unsurprising. The work described in this chapter showed that the transcriptional activity of the whole CpG island region, rather than individual exons 1, was down-regulated by dexamethasone. As such, the observation that down-regulation of GR expression reaches a 'ceiling', after which further glucocorticoid treatment causes no further decrease, cannot be explained by the selective down-regulation of one or more alternate exons 1, leaving the other exons to maintain a basal level of GR expression. Therefore, in the same way that tissue-specific regulation of rat GR does not appear to occur through the selective promoter activity of one or more exons 1, the mechanism of autoregulation does not operate on specific, individual exons 1.

7 Discussion

The work described in this thesis was designed to investigate the functional relevance of the rat glucocorticoid receptor (GR) promoter region. Specifically, the involvement of the multiple exon 1 structure of the GR promoter in tissue-specific, constitutive and auto-regulation was studied.

The importance of maintaining appropriate GR levels in every cell has been well-documented (see section 1.2.2.2). Similarly, the complex regulation of the GR gene, in which levels vary both between and within tissues, has been the subject of much investigation. The GR promoter shows a great deal of complexity, consisting of multiple exons 1 that are alternatively spliced onto a common exon 2. However, an in-frame translational stop codon downstream of the splice acceptor site in exon 2 means that the sequence of the translated protein is unaffected by the first exon. As such, all GR transcripts encode a common protein, but differ in their 5' leader sequence. While most of the alternate exons 1 are located within a ~3.2 kb region containing two CpG islands, exon 1_1 lies ~30.2kb upstream of exon 2. This multiple alternate exon 1 structure naturally suggests itself as a possible mechanism for providing complex GR regulation.

It is conceivable that tissue-specific patterns of alternate exon 1 expression might provide a means by which transcriptional regulation of GR is exerted in response to cell-specific factors. This hypothesis implies that alternate exons 1 are expressed in a tissue-specific manner. RPA analysis carried out in this laboratory showed that, while exon 1_6 and 1_{10}-containing transcripts predominated and were present in every rat tissue examined, transcripts containing exons 1_5 and 1_7 were only detectable in hippocampus (both at ~8%) (McCormick et al., 2000). However, the more sensitive RT-PCR analysis used in this investigation showed that all alternate GR exons 1 located in the CpG island region (1_4, 1_5, 1_6, 1_7, 1_{10} and 1_{11}) were expressed in all rodent cell lines tested.

These data are consistent with further RT-PCR analysis carried out in this laboratory, in which all 'CpG island' alternate exons 1 were detected in all rat tissues tested (Freeman, 2003). Similarly, studies of human GR have shown exons 1B and 1C (homologous to rat exons 1_6 and 1_{10} respectively) to be ubiquitously expressed (Breslin et al., 2001; Nunez & Vedeckis, 2002). Mouse GR exons 1B, 1C, 1D and 1E (the last two being homologous to rat exons 1_5 and 1_{11} respectively) have also been detected in every mouse tissue tested (Strahle et al., 1992; Chen et al., 1999).

This contrasts with results showing that expression of exon 1_1, which lies outside the CpG island region, ~30.2kb upstream of exon 2, is tissue-specific. Previous RPA analysis showed that exon 1_1-containing transcripts were found exclusively in rat thymus, where they accounted for ~25% of total GR mRNA (McCormick et al., 2000; McCormick, 2000). RT-PCR analysis of rodent cells lines carried out during this investigation confirmed that exon 1_1 expression was only detectable in the mouse T cell lines S-49 and EL-4. These data are consistent with RT-PCR results carried out rat tissues, which also showed that exon 1_1 expression was restricted to the thymus (Freeman, 2003). However, studies in mouse showed that the 1A transcript was present in all tissues investigated (Chen et al., 1999). Furthermore, three alternate 1A transcripts, termed 1A1, 1A2 and 1A3 have been described for human GR and while expression of 1A3 appears to be restricted to cells of the immune system, 1A1 and 1A2 are ubiquitously expressed (Breslin et al., 2001). Further investigation of GR exon 1_1 regulation, particularly with regard to whether alternate 1_1 variants are conserved in the rat, is required and is currently being undertaken in this laboratory.

It appears therefore that cells do not operate an 'all-or-nothing' policy with regard to expression of the GR exons 1 residing within the CpG island region. Rather, transcription is initiated at all 'CpG island' exons 1 in all cells. Regulation of GR does not occur as a result of switching the transcription of alternate exons 1 'on' or 'off'. If the GR 'CpG island' exons do indeed play a role in the regulation of GR, it must be through adjustment of their relative levels.

In this way, the multiple alternate exons 1 of the CpG island region might be driven by multiple promoters that show differential activity between cell types. As such, transient transfection experiments involving constructs in which fragments of the rat GR promoter were fused to a luciferase reporter gene were carried out in B103 (rat neuroblastoma) and H4IIE (rat hepatoma) cells. B103 cells were chosen based on previous work showing that a construct associated with the exon 1_7 exhibited neural-specific promoter activity (McCormick et al., 2000; McCormick, 2000). In addition, H4IIE cells were found to express both alternate exons 1 and total GR mRNA at levels comparable with rat liver. The P2 construct, which represented the whole of the rat GR promoter, showed the highest activity in both cell types. Transient transfections using a 3' deletion series of the GR promoter, designed to measure the activity associated with each individual exon 1 (McCormick et al., 2000; McCormick, 2000), confirmed that $P1_7$ was the only construct associated with cell-specific promoter activity, being restricted to neural cells.

A 5' deletion series of $P1_7$ identified a 134bp *PstI* fragment necessary for high neural-specific promoter activity. Furthermore, deletion of this fragment from P2 caused a neural-specific decrease in promoter activity. Interestingly, the high activity associated with this fragment was lost in the reverse orientation, suggesting a lack of classical enhancer properties. Consistent with this, the fragment did not activate a heterologous promoter, but did show orientation-specific activity within a promoterless vector, which was higher in B103 than H4IIE cells. This directionality was also observed during *DNaseI* protection experiments, in which both B103 and H4IIE extracts protected a footprint near the 3' end of the 134bp *PstI* fragment, which was detectable on one strand only. Comparison of the protected sequence with the TRANSFAC database showed it to encode a putative AP2 site. However, further *DNaseI* protection and EMSA analyses failed to provide evidence of AP2 binding. While EMSAs did show evidence that Sp1 or an Sp1-related protein, present in both B103 and H4IIE cell extracts, bound to the 134bp *PstI* fragment, it remains to be determined if this binding is of sufficiently high affinity to be physiologically relevant. Importantly, the observed binding was not specific to B103 cells and therefore did not provide a candidate factor that might be responsible for the high

neural-specific activity of this region. The directionality of the observed binding also remains unexplained. It may be the case that the 134bp *PstI* fragment contains a transcription initiation region that is more active in neural cells, although EMSAs showed no evidence of TFIID-specific binding (TFIID is a component of the initiation complex).

Interestingly, apart from 1_7, no other exon 1 was associated with cell-specific promoter activity. Indeed, $P1_7$ and $P1_{9/10}$ were the only constructs to show significant promoter activity in any cell type. In light of evidence showing that exon 1_6- and 1_{10}-containing transcripts account for the vast majority of GR mRNA (McCormick et al., 2000; McCormick, 2000), the lack of promoter activity associated with $P1_6$ and $P1_{10}$ was particularly surprising. This result also contrasted with previous work showing significant promoter activity associated with the human GR exons 1B and 1C (homologous to rat exons 1_6 and 1_{10} respectively) (Breslin et al., 2001; Nunez & Vedeckis, 2002). Indeed, *DNaseI* footprinting, EMSAs and deletion analyses have implicated binding of transcription factors including YY1, Sp1 and AP2 in the tissue-specific promoter activity of exons 1B and 1C (Nobukuni et al., 1995; Breslin et al., 2001; Nunez & Vedeckis, 2002). The published data regarding potential *cis*-regulatory elements of the GR promoter suggest that tissue-specific expression of alternate GR exons 1 is not controlled by the action of tissue-specific transcription factors. Rather, combinations of ubiquitously expressed transcription factors, e.g. YY1 and Sp1, act to control activity over the whole promoter, including that associated with exons 1B and 1C. For instance, in HeLa cells, deletion of three YY1 sites from the 1B promoter causes only a 30% loss in activity, while a substantial decrease in activity is observed following deletion of all four Sp1 sites (Nunez & Vedeckis, 2002). In contrast, the elimination of the same three YY1 sites results in a dramatic reduction in promoter activity in both HepG2 and Jurkat cells (64 and 77% respectively), while deletion of the Sp1 sites appears to have no effect (Nunez & Vedeckis, 2002).

However, these data also suggest that the relative contribution of these regulatory elements is context-dependent. For example, while deletion of the four YY1 sites

from the isolated 1B promoter resulted in only a ~30% decrease in activity in HeLa cells (Nunez & Vedeckis, 2002), their deletion from a construct representing the whole GR promoter led to a ~60% reduction in activity (Breslin & Vedeckis, 1998). This indicates that transcription from alternate exons 1 may be dependent upon their context and position within the whole promoter, suggesting that isolation of discrete fragments may not provide an accurate picture of the regulatory elements involved. In this way, differences in the size and relative position of constructs representing promoters 1_6 and 1_{10} in this study and the 1B and 1C constructs used by Nunez *et al* (Nunez & Vedeckis, 2002) and Breslin *et al* (Breslin & Vedeckis, 1998) may explain the discrepancy in observed activities.

The suggestion that regulatory regions are context-dependent also has implications for results relating to the activity of the 134bp *PstI* fragment. Transfection data showed that the contribution of this fragment generated significantly higher activity in the context of $P1_{7b}$ than when isolated in front of a promoterless vector. It is therefore possible that binding at the 134bp *PstI* fragment might be enhanced by regulatory elements located outside the region. The contrast between the insignificant promoter activity associated with $P1_6$ and $P1_{10}$ found during this investigation and the predominance of exon 1_6 and 1_{10}-containing transcripts reported in rat tissues (McCormick et al., 2000; McCormick, 2000) may also be explained by this contextual dependence. That is, regulatory elements present in the whole promoter, but not present in $P1_6$ or $P1_{10}$, may increase the transcriptional activity of exons 1_6 and 1_{10}.

The idea that activity of discrete elements in the GR promoter is dependent upon their relative position and context within the whole region was enhanced by the findings of experiments using the GR 5' deletion series. Results showed that while the loss of sections between –4878 to –3339 and –1770 to -9 caused significant decreases in activity in B103 cells, the presence of these regions in other constructs, including $P1_6$ and pVL262, was not associated with significant activity. It therefore appears that these regions are necessary, but not sufficient for high GR promoter activity in B103 cells.

The balance of evidence gained from this study and others therefore suggests that transcription from alternate GR exons 1 is not directed by discrete exon 1-associated promoters that act in isolation. Instead, transcriptional activity at alternate GR exons 1 is dependent upon regulatory interactions over the whole promoter. As such, a model can be proposed in which individual exons 1 are not accompanied by individual promoters; rather, transcription is initiated from multiple preferential sites (giving rise to multiple exons 1) that lie within a large promoter 'region' (fig.7.1). Under this model, the selection of individual exon 1 transcription initiation sites is not of primary importance. Instead, transcription initiation at alternate exons 1 is simply a consequence of the combined activity of regulatory elements positioned over the whole promoter.

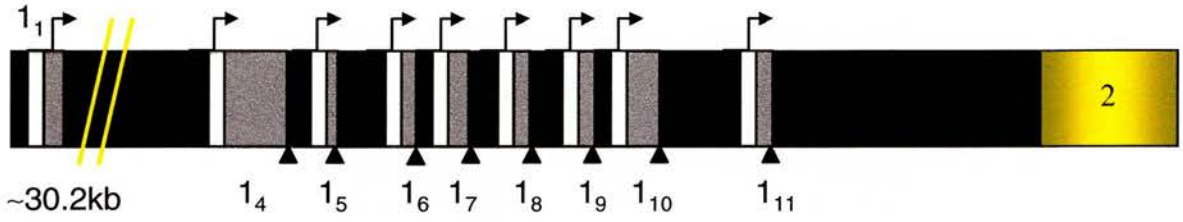
Several pieces of evidence gained during this investigation support this model. Under this hypothesis, the splice donor sites associated with each exon 1 are of minimal importance; the transcript is simply spliced at the next suitable donor site following the initiation point. Consistent with this, transient transfections of P2 constructs carrying mutations at the splice donor sites of either exons 1_6, 1_{10} or 1_{11}, followed by RT-PCR and sequence analysis, identified longer transcripts that were indeed spliced at donor sites downstream of the mutations. Furthermore, mutagenesis of these sites was not associated with any decrease in promoter activity.

If relatively indiscriminate transcription initiation is indeed occurring over the whole CpG island region, this extended area of transcriptional activity should be accompanied by a corresponding region of 'open' chromatin. Accordingly, *DNaseI* hypersensitive site mapping identified a ~3kb region of transcriptionally active chromatin, bounded by hypersensitive sites, that extended over the whole CpG island region.

However, while transcriptional regulation of GR may not occur through the selection of individual exon 1 transcription initiation sites, there are several advantages in driving transcription from multiple start sites, particularly with regard to ensuring

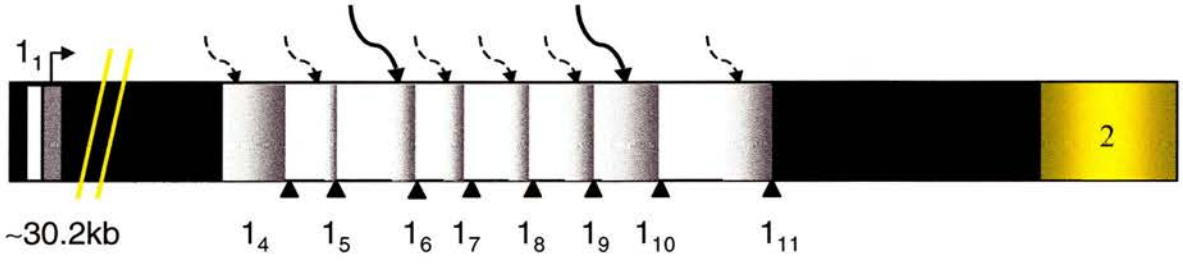
Figure 7.1 *Alternative models of GR promoter activity over the CpG island region.* Diagram showing two alternative models of promoter activity over the GR CpG island region. Both exon 2 and exon 1_1, which lies ~30.2kb upstream of exon 2, are indicated. Mapped splice donor sites are marked with a triangle.
A: Each alternate exon 1 (shown in grey) is accompanied by an individual promoter (shown in white). Transcription from each individual promoter is indicated by a corner arrow.
B: The promoter 'region' is shown in white, within which transcription from multiple initiation sites (labelled with wavy arrows) gives rise to multiple exons 1 (shown in grey). Larger arrows are used to indicate transcription initiation from the two most predominant exons, 1_6 and 1_{10}.

A



CpG Island Region

B



CpG Island Region

constitutive expression of this essential housekeeping gene. The data described here present the intriguing possibility that GR expression, in which transcription is indiscriminately initiated at several sites, might be rendered less vulnerable to mutations. The mutagenesis experiments performed during this study show that the GR promoter exhibits a great deal of resistance to mutation, even if the disruption is not 'random' but directed at the splice donor sites crucial in maintaining 'normal' exon 1 structure. This robustness would obviously be of enormous benefit to a housekeeping gene such as GR, whose expression must be guaranteed in every cell.

Furthermore, the dual role of CpG islands as promoters and DNA replication origins causes them to be under a greater mutational pressure than the rest of the genome (Delgado et al., 1998; Antequera & Bird, 1999; Cuadrado et al., 2001). As such, maintaining ubiquitous GR expression in the face of this large mutational burden may require the protection of multiple sites of transcription initiation.

I therefore suggest that the primary function of alternate GR exons 1 within the CpG island region is not one of tissue-specific regulation, but rather a way of ensuring constitutive GR expression. In this way, individual exons 1 are not accompanied by individual promoters that are selectively regulated in different tissues. Instead, tissue-specific regulation occurs through interactions over the whole promoter 'region', giving rise to characteristic exon 1 expression patterns. Furthermore, it appears that after characteristic patterns of GR exon 1 expression are 'set' in different tissues, up- or down-regulation of GR transcription does not act selectively at individual exons 1, but on this pattern as a whole.

This proposal is supported by results gained from investigations into autoregulation of the GR promoter. Strikingly, all constructs in the 3' deletion series, which measures the activity associated with each individual exon 1, showed the same degree of repression. It appears, therefore, that autoregulation does not operate by repressing the expression of one or more individual exons 1 while leaving the others unaffected, or indeed by down-regulating individual exon 1 activity to different extents. This finding has been confirmed by *in vivo* work carried out in this

laboratory, in which *in situ* hybridisation analysis of rat liver showed that individual GR exons 1 were not selectively down regulated in dexamethasone-treated animals (Freeman, 2003). Furthermore, RPA analyses of liver taken from dexamethasone-treated rats showed that characteristic 'patterns' of GR exon 1 expression are maintained following down-regulation (Freeman, 2003).

The number of characterised genes containing multiple promoters has increased rapidly over the last decade. Many studies have speculated on the advantages gained by such a transcriptional system, with the prevailing view that transcription from multiple promoters permits additional flexibility in the control of gene expression (Ayoubi & Van De Ven, 1996). In this way, multiple promoter regions can be seen to provide all the regulatory elements needed for tissue- and/or development-specific expression, as well as allowing responsiveness to hormonal and/or nutritional stimuli. This was thought to be the case for GR, in which each alternate exon 1 was supposed to be driven by an individual promoter, thus providing the required flexibility of GR expression. However, results presented in this thesis provided an alternative hypothesis. Under this model, the CpG island region of the GR promoter occupies an extended area of active chromatin in which transcription occurs at heterogeneous transcription initiation sites that give rise to multiple alternate exons 1. These alternate exons 1 are not accompanied by individual discrete promoters but rather form part of a large promoter 'region'. As such, cells do not express specific combinations of alternate exons 1; instead, all exons 1 are expressed in all cells. Combinations of ubiquitous transcription factors, most probably including YY1 and Sp1, act at regulatory elements located over the whole promoter region to drive this heterogeneous transcription. Tissue-specific levels of GR transcriptional activity are regulated by these ubiquitous transcription factor combinations, which do not act selectively at specific exons 1, but may produce tissue-specific 'patterns' of relative exon 1 expression. This combined activity means that regional regulatory elements are position- and context- dependent with regard to the whole promoter region. Regulation of GR promoter activity is therefore not dependent upon the selection of individual exon 1 transcription start or, indeed, splice donor sites. Similarly, autoregulation of GR transcriptional activity does not occur in an exon 1-specific

manner but instead dampens activity over the whole promoter region. However, while selection of alternate exon 1 transcription initiation sites may not be of primary importance to the tissue-specific or auto-regulation of GR, the multiple exon 1 structure does ensure that promoter activity is especially robust to mutations and thus ensures the constitutive expression of this vital housekeeping gene.

Appendix I: Addresses of Suppliers

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks., HP7 9NA, U.K.

Beckman Coulter (U.K.) Ltd., Oakley Court, Kingsmead Business Park, London Road, High Wycombe, Bucks., HP11 1JU, U.K.

Berthold Technologies (U.K.) Ltd., The Priory, High Street, Redbourn, Herts., AL3 7LZ, U.K.

BD UK Ltd, Between Towns Road, Cowley, Oxford, Oxon., OX4 3LY, U.K.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts., HP2 7TD, U.K.

Cambrex Bio Science Wokingham Ltd., 1 Ashville Way, Wokingham, Berks., RG41 2PL, U.K.

Eppendorf AG, Barkhausenweg 1, 22331 Hamburg, Germany

Greiner Bio-One Ltd., Brunel Way, Stroudwater Business Park, Stonehouse, Glos., GL10 3SX, U.K.

IKA Labortechnik, IKA-Werke GmbH & Co. KG, Janke & Kunkel-Str. 10, D-79219 Staufen, Germany

Invitrogen, Ichinnan Business Park, 3 Fountain Drive, Paisley, PA4 9RF, U.K.

Kendro Laboratory Products Plc., Stortford Hall Park, Bishhop's Stortford, Herts., CM23 5GZ, U.K.

Kodak Ltd., Hemel Hempstead, Herts., U.K.

Nalgene Labware, Unit 1a, Thorn Business Park, Hereford, HR2 6JS, U.K.

Oswel DNA service, Medical & Biological Sciences Building, University of Southampton, Boldrewood, Bassett Crescent East, Southampton, SO16 7PX, UK.

Promega Ltd., Delta house, Chilworth Research Centre, Southampton, SO1 7NS, U.K.

Qbiogene-Alexis Ltd., P.O. Box 6757, Bingham, Nottingham, NG13 8LS, U.K.

Quantum Appligene, Parc d'Innovation, BP 72, Illkirch 67402, France

Roche Diagnostics Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, U.K.

Sarstedt Ltd., 68 Boston Road, Beaumont Leys, Leicester, LE4 1AW, U.K.

Shimadzu Europa U.K., Mill Court, Featherstone Road, Wolverton Mill South, Milton Keynes, Bucks., MK12 5RE, U.K.

Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4OH, U.K.

Stratagene Europe, Gebouw California, Hogehilweg 15, 1101 CB Amsterdam Zuidoost, The Netherlands

Techne, Duxford, Cambridge, CB2 4P2, U.K.

Tropix Ltd., 47 Wiggins Avenue, Bedford, MA, U.S.A.

Worthington Biochemicals, Lorne Laboratories Ltd., 7 Tavistock Estate, Ruscombe Business Park, Ruscombe Lane, Twyford, Reading, Berks., RG10 9NJ, U.K.

VWR International Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leics., LE17 4XN, U.K.

Appendix II: The rat GR promoter sequence

The sequence of 5'-DNA flanking exon 2 of the rat GR gene is shown below. Numbering is with respect to the translation start, +1. Underlined sequence in red indicate exon 1 sequences found in 5'-RACE PCR clones amplified from hippocampal GR mRNA. In the case of exon 16 (which was not represented among the 5'-RACE clones), the red underlined nucleotides are those present in the published rat cDNA sequence (Miesfeld, 1984). Blue sequences represent primers used in GR exon 1 specific PCR reactions.The start of exon 2 is at -13. Adapted from McCormick et al., 2000.

```
-4600  TAGTATAGGTTTTCCTTCTTGAGGTATCAAGCTTCTATTCCTTTGCCAAGATGGCTGCCCTGGATCCCATGGAGGTAGCGACCGTGCGGCATCTCTGCCC
-4500  AAGGAGCCCGCTTACAGTCACGTTCTCCCCGTGCAAAGCGGACGATACATTGGGCAGCCTTTAAGCTTTTCATCCAAGAAAGAACGACTCGGGTTTGACG
-4400  CCAAAGAGCACCTTTGCCAAGATGGTGACCGTGCGGCGTCACTGCTCTTTACCAAGATGGCGGCGAGGGACTTCCGGCACGCGCTTCCCCAATCAGGGAT
-4300  CTCCAAGAGGTCAGGCAGAGGAGACCGCCCTTGGAGTCGAAGTGCGGCGCGAGCCGCTTCTGCCGCCCGCCGCGGGAGGGCATGAGGTGGAGTCATGGCC
1_4 -4200  ACCTCCGCTCTAATCAGAAGTGCCAAGCGCTGGCACCTGTGGGGGAGCAAAAGTTACTTCCTTGCACCCCAAAGCAACACCGTAACACCTTTCCCGGAGT
-4100  CCCCCAAATCCTAGCCTATGGCATGAGGTAGAGGGGCACGGTCCCGGCGTCGCCCCAGCCTGCCTAACACAGAGGACACCCGAGGGGCAGGTACTGCGCG
-4000  GCCGCACAGTCACCCTACGCCCCTTTCCTGTCCTAGGGGGACCGGCCATGTGTTTCTCTTGGAGACCCGGGGACTCGTATTGGGCACAGCTGGACGGAGC
-3900  TAAAAGCTGACGTTTTAAAGATGCATGTTTTTGTTTTATTTGGAGGGACAGAGGGGTCCCTGGAACCCAGAAAGCTGAGCAAGGCACCGAGCCTGGAGCA
-3800  GCAAATGTCAAGATTCGGGGGAGGGGCCTCCGCGGGGAGCTTGGATGCTGGCCCCGAAGGGGGTGGAAGGAGAGGTCAGGAGTTTGGGTAAGAGGAGGGC
-3700  GGACTTCAGCAGCAACTTACTATTCGGTCTGCAACTTGCTTCTAGGCCTGCACACACCCCCTCCCGCCCCGCAAGGCTTCCTTAATCACAATTTTTTTTT
1_5 -3600  TAAGTGCAAAGAAACCCAGCTCTCTGAGAGGGTTTTGCATTCGGCATGCAACTTCCTCCGAGTGTGAGCGCGCTGGCAGGCGGGGAGGGAGCGGTGGGGG
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Reference List

Adams M, Meijer OC, Wang J, Bhargava A, Pearce D 2003 Homodimerization of the Glucocorticoid Receptor is not Essential for Response Element Binding: Activation of the Phenylethanolamine N-Methyltransferase Gene by Dimerization-Defective Mutants. *Mol Endocrinol* (in press)

Akana SF, Cascio CS, Shinsako J, Dallman MF 1985 Corticosterone: narrow range required for normal body and thymus weight and ACTH. *Am J Physiol* **249**:t-32

Alksnis M, Barkhem T, Stromstedt P-E, Ahola H, Kutoh E, Gustafsson JA, Poellinger L, Nilsson S. 1991. High level expression of functional full length and truncated glucocorticoid receptor in chinese hamster ovary cells: demonstration of ligand-induced down-regulation of expressed receptor messenger RNA and protein. *J Biol Chem* **266**, 10078-10085.

Almawi WY, Tamim H 2001 Posttranscriptional mechanisms of glucocorticoid antiproliferative effects: glucocorticoids inhibit IL-6-induced proliferation of B9 hybridoma cells. *Cell Transplant* **10**:161-164

Ambrosio R, Fimiani G, Monfregola J, Sanzari E, De Felice N, Salerno MC, Pignata C, D'Urso M, Ursini MV 2002 The structure of human STAT5A and B genes reveals two regions of nearly identical sequence and an alternative tissue specific STAT5B promoter. *Gene* **285**:311-318

Anderson DJ 1993 Cell and molecular biology of neural crest cell lineage diversification. *Curr Opin Neurobiol* **3**:8-13

Ansari AZ, Bradner JE, O'Halloran TV 1995 DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374**:371-375

Antequera F, Boyes J, Bird A 1990 High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **62**:503-514

Antequera F, Bird A 1993 Number of CpG islands and genes in human and mouse. *Proc Natl Acad Sci U S A* **90**:11995-11999

Antequera F, Bird A 1999 CpG islands as genomic footprints of promoters that are associated with replication origins. *Curr Biol* **9**:R661-R667

Arriza JL, Simerly RB, Swanson LW, Evans RM 1988 The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* **1**:887-900

Ashwell JD, Lu FW, Vacchio MS 2000 Glucocorticoids in T cell development and function. *Annu Rev Immunol* **18**:309-345

Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M 1995 Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* **270**:286-290

Ayoubi TA, Van De Ven WJ 1996 Regulation of gene expression by alternative promoters. **FASEB J** *10*:453-460

Ballard PL, Baxter JD, Higgins SJ, Rousseau GG, Tomkins GM 1974 General presence of glucocorticoid receptors in mammalian tissues. *Endocrinology* **94**:998-1002

Bamberger CM, Bamberger AM, de Castro M, Chrousos GP 1995 Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest* **95**:2435-2441

Barker DJ 1990 The fetal and infant origins of adult disease. *BMJ* **301**:1111

Barker DJ, Bull AR, Osmond C, Simmonds SJ 1990 Fetal and placental size and risk of hypertension in adult life. *BMJ* **301**:259-262

Barker DJ 1997 Fetal nutrition and cardiovascular disease in later life. *Br Med Bull* **53**:96-108

Barnes PJ, Adcock IM 1998 Transcription factors and asthma. *Eur Respir J* **12**:221-234

Barradeau S, Imaizumi-Scherrer T, Weiss MC, Faust DM 2000 Alternative 5'-exons of the mouse cAMP-dependent protein kinase subunit RIalpha gene are conserved and expressed in both a ubiquitous and tissue-restricted fashion. *FEBS Lett* **476**:272-276

Bauer A, Tronche F, Wessely O, Kellendonk C, Reichardt HM, Steinlein P, Schutz G, Beug H 1999 The glucocorticoid receptor is required for stress erythropoiesis. *Genes Dev* **13**:2996-3002

Baumann H, Jahreis GP, Morella KK 1990 Interaction of cytokine- and glucocorticoid-response elements of acute-phase plasma protein genes. Importance of glucocorticoid receptor level and cell type for regulation of the elements from rat alpha 1-acid glycoprotein and beta-fibrinogen genes. *J Biol Chem* **265**:22275-22281

Beato M, Herrlich P, Schutz G 1995 Steroid hormone receptors: many actors in search of a plot. *Cell* **83**:851-857

Beato M, Chavez S, Truss M 1996 Transcriptional regulation by steroid hormones. **Steroids 61**:240-251

Becker M, Baumann C, John S, Walker DA, Vigneron M, McNally JG, Hager GL 2002 Dynamic behavior of transcription factors on a natural promoter in living cells. *EMBO Rep* **3**:1188-1194

Bird A. 1984 DNA methylation - how important in gene control? *Nature* **307**, 503-504.

Bird AP 1986 CpG-rich islands and the function of DNA methylation. *Nature* **321**:209-213

Black DL 2003 Mechanisms of Alternative Pre-Messenger RNA Splicing. *Annu Rev Biochem* (in press)

Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE 2002 Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110**:93-105

Blin N, Stafford DW 1976 A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res* **3**:2303-2308

Bodwell JE, Orti E, Coull JM, Pappin DJ, Smith LI, Swift F 1991 Identification of phosphorylated sites in the mouse glucocorticoid receptor. *J Biol Chem* **266**:7549-7555

Bodwell JE, Webster JC, Jewell CM, Cidlowski JA, Hu JM, Munck A 1998 Glucocorticoid receptor phosphorylation: overview, function and cell cycle-dependence. *J Steroid Biochem Mol Biol* **65**:91-99

Bonham K, Ritchie SA, Dehm SM, Snyder K, Boyd FM 2000 An alternative, human SRC promoter and its regulation by hepatic nuclear factor-1alpha. *J Biol Chem* **275**:37604-37611

Borski RJ 2000 Nongenomic membrane actions of glucocorticoids in vertebrates. *Trends Endocrinol Metab* **11**:427-436

Brandon DD, Kendall JW, Alman K, Tower P, Loriaux DL 1995 Inhibition of dexamethasone binding to human glucocorticoid receptor by New World primate cell extracts. *Steroids* **60**:463-466

Breslin MB, Vedeckis WV 1998 The human glucocorticoid receptor promoter upstream sequences contain binding sites for the ubiquitous transcription factor, Yin Yang 1. J *Steroid Biochem Mol Biol* **67**:369-381

Breslin MB, Geng CD, Vedeckis WV 2001 Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. *Mol Endocrinol* **15**:1381-1395

Brindley DN 1995 Role of glucocorticoids and fatty acids in the impairment of lipid metabolism observed in the metabolic syndrome. *Int J Obes Relat Metab Disord* **19** Suppl 1:S69-75

Burnstein KL, Bellingham DL, Jewell CM, Powell-Oliver FE, Cidlowski JA 1991 Autoregulation of glucocorticoid receptor gene expression. *Steroids* **56**:52-58

Burnstein KL, Jewell CM, Sar M, Cidlowski JA 1994 Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone-inducible receptor messenger RNA down-regulation through multiple mechanisms. *Mol Endocrinol* **8**:1764-1773

Cadepond F, Schweizer-Groyer G, Segard-Maurel I, Jibard N, Hollenberg SM, Giguere V, Evans RM, Baulieu EE 1991 Heat shock protein 90 as a critical factor in maintaining glucocorticosteroid receptor in a nonfunctional state. *J Biol Chem* **266**:5834-5841

Chen F, Watson CS, Gametchu B 1999a Association of the glucocorticoid receptor alternatively-spliced transcript 1A with the presence of the high molecular weight membrane glucocorticoid receptor in mouse lymphoma cells. *J Cell Biochem* **74**:430-446

Chen F, Watson CS, Gametchu B 1999b Multiple glucocorticoid receptor transcripts in membrane glucocorticoid receptor-enriched S-49 mouse lymphoma cells. *J Cell Biochem* **74**:418-429

Cheung WL, Briggs SD, Allis CD 2000 Acetylation and chromosomal functions. *Curr Opin Cell Biol* **12**:326-333

Chretien S, Dubart A, Beaupain D, Raich N, Grandchamp B, Rosa J, Goossens M, Romeo PH 1988 Alternative transcription and splicing of the human porphobilinogen deaminase gene result either in tissue-specific or in housekeeping expression. *Proc Natl Acad Sci U S A* **85**:6-10

Christodoulopoulos P, Leung DY, Elliott MW, Hogg JC, Muro S, Toda M, Laberge S, Hamid QA 2000 Increased number of glucocorticoid receptor-beta-expressing cells in the airways in fatal asthma. *J Allergy Clin Immunol* **106**:479-484

Cidlowski JA, Cidlowski NB 1982 Glucocorticoid receptors and the cell cycle: evidence that the accumulation of glucocorticoid receptors during the S phase of the cell cycle is dependent on ribonucleic acid and protein synthesis. *Endocrinology* **110**:1653-1662

Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schutz G 1995 Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev* **9**:1608-1621

Cole TJ, Myles K, Purton JF, Brereton PS, Solomon NM, Godfrey DI, Funder JW 2001 GRKO mice express an aberrant dexamethasone-binding glucocorticoid receptor, but are profoundly glucocorticoid resistant. *Mol Cell Endocrinol* **173**:193-202

Crocker IC, Church MK, Newton S, Townley RG 1998 Glucocorticoids inhibit proliferation and interleukin-4 and interleukin-5 secretion by aeroallergen-specific T-helper type 2 cell lines. *Ann Allergy Asthma Immunol* **80**:509-516

Cuadrado M, Sacristan M, Antequera F 2001 Species-specific organisation of CpG island promoters at mammalian homologous genes. *EMBO Rep* **2**:586-592.

Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J 1991 Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. *J Biol Chem* **266**:3107-3112

Dallman MF, Akana SF, Strack AM, Hanson ES, Sebastian RJ 1995 The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons. *Ann N Y Acad Sci* **771**:730-742

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M 1998 Brain corticosteroid receptor balance in health and disease. *Endocr Rev* **19**:269-301

de Lange P, Koper JW, Brinkmann AO, de Jong FH, Lamberts SW 1999 Natural variants of the beta isoform of the human glucocorticoid receptor do not alter sensitivity to glucocorticoids. *Mol Cell Endocrinol* **153**:163-168

Delgado S, Gomez M, Bird A, Antequera F 1998 Initiation of DNA replication at CpG islands in mammalian chromosomes. *EMBO J* **17**:2426-2435

Denton RR, Eisen LP, Elsasser MS, Harmon JM 1993 Differential autoregulation of glucocorticoid receptor expression in human T- and B-cell lines. *Endocrinology* **133**:248-256

DeRijk RH, Schaaf M, De Kloet ER 2002 Glucocorticoid receptor variants: clinical implications. *J Steroid Biochem Mol Biol* **81**:103-122

Deroo BJ, Archer TK 2001 Glucocorticoid receptor-mediated chromatin remodeling in vivo. *Oncogene* **20**:3039-3046

de Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S 1987 Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* **7**:725-737

Diba F, Watson CS, Gametchu B 2001 5'UTR sequences of the glucocorticoid receptor 1A transcript encode a peptide associated with translational regulation of the glucocorticoid receptor. *J Cell Biochem* **81**:149-161

Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**:1475-1489

Dominski Z, Kole R 1994 Identification of exon sequences involved in splice site selection. *J Biol Chem* **269**:23590-23596

Dong Y, Poellinger L, Gustafsson JA, Okret S 1988 Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. *Mol Endocrinol* **2**:1256-1264

Drouin J, Trifiro MA, Plante RK, Nemer M, Eriksson P, Wrange O 1989 Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. *Mol Cell Biol* **9**:5305-5314

Dunn JF, Nisula BC, Rodbard D 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J Clin Endocrinol Metab* **53**:58-68

Eisen LP, Elsasser MS, Harmon JM 1988 Positive regulation of the glucocorticoid receptor in human T-cells sensitive to the cytolytic effects of glucocorticoids. *J Biol Chem* **263**:12044-12048

Elenkov IJ, Chrousos GP 1999 Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease. *Trends Endocrinol Metab* **10**:359-368

Encio IJ, Detera-Wadleigh SD 1991 The genomic structure of the human glucocorticoid receptor. *J Biol Chem* **266**:7182-7188

Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Bruhl B, Beier K, Metz J, Garcia-Arraras JE, Roig-Lopez JL, Monaghan P, Schmid W, Cole TJ, Kellendonk C, Tronche F, Schutz G, Unsicker K 1999 Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. *Development* **126**:2935-2944

Flaherty L, Rinchik E 1978 No evidence for foreign H-2 specificities on the EL4 mouse lymphoma. *Nature* **273**:52-53

Flouriot G, Griffin C, Kenealy M, Sonntag-Buck V, Gannon F 1998 Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. *Mol Endocrinol* **12**:1939-1954

Freedman LP 1999 Multimeric Coactivator Complexes for Steroid/Nuclear Receptors. *Trends Endocrinol Metab* **10**:403-407

Freeman A. 2002 5' variants glucocorticoid receptor mRNA: further studies of tissue-specificity and regulation. *PhD thesis.* University of Edinburgh

Fryer CJ, Archer TK 1998 Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature* **393**:88-91

Fukuda K, Higashida H, Kubo T, Maeda A, Akiba I, Bujo H, Mishina M, Numa S 1988 Selective coupling with K+ currents of muscarinic acetylcholine receptor subtypes in NG108-15 cells. *Nature* **335**:355-358

Funder JW 1992 Glucocorticoid receptors. *J Steroid Biochem Mol Biol* **43**:389-394

Galas DJ, Schmitz A 1978 DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res* **5**:3157-3170

Gametchu B, Watson CS, Pasko D 1991a Size and steroid-binding characterization of membrane-associated glucocorticoid receptor in S-49 lymphoma cells. *Steroids* **56**:402-410

Gametchu B, Watson CS, Shih CC, Dashew B 1991b Studies on the arrangement of glucocorticoid receptors in the plasma membrane of S-49 lymphoma cells. *Steroids* **56**:411-419

Garland RC 1986 Induction of glucose 6-phosphatase in cultured hepatoma cells by dexamethasone. *Biochem Biophys Res Commun* **139**:1130-1134

Gass P, Reichardt HM, Strekalova T, Henn F, Tronche F 2001 Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: models for depression and anxiety? *Physiol Behav* **73**:811-825

Gays F, Unnikrishnan M, Shrestha S, Fraser KP, Brown AR, Tristram CM, Chrzanowska-Lightowlers ZM, Brooks CG 2000 The mouse tumor cell lines EL4 and RMA display mosaic expression of NK-related and certain other surface molecules and appear to have a common origin. *J Immunol* **164**:5094-5102

Gearing KL, Cairns W, Okret S, Gustafsson JA 1993 Heterogeneity in the 5' untranslated region of the rat glucocorticoid receptor mRNA. *J Steroid Biochem Mol Biol* **46**:635-639

Gehring U, Segnitz B, Foellmer B, Francke U 1985 Assignment of the human gene for the glucocorticoid receptor to chromosome 5. *Proc Natl Acad Sci U S A* **82**:3751-3755

Gidoni D, Dynan WS, Tjian R 1984 Multiple specific contacts between a mammalian transcription factor and its cognate promoters. *Nature* **312**:409-413

Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM 1986 Functional domains of the human glucocorticoid receptor. *Cell* **46**:645-652

Gill G. 2001 Regulation of the initiation of eukaryotic transcription. *Essays in Biochemistry* vol 37: pp33-43. Edited by Chapman KE and Higgins SJ.

Godfrey DI, Purton JF, Boyd RL, Cole TJ 2000 Stress-free T-cell development: glucocorticoids are not obligatory. *Immunol Today* **21**:606-611

Godfrey DI, Purton JF, Boyd RL, Cole TJ 2001 Glucocorticoids and the thymus: the view from the middle of the road. *Trends Immunol* **22**:243

Gomi M, Moriwaki K, Katagiri S, Kurata Y, Thompson EB 1990 Glucocorticoid effects on myeloma cells in culture: correlation of growth inhibition with induction of glucocorticoid receptor messenger RNA. *Cancer Res* **50**:1873-1878

Govindan MV, Pothier F, Leclerc S, Palaniswami R, Xie B 1991 Human glucocorticoid receptor gene promotor - homologous down regulation. *J Steroid Biochem Mol Biol* **40**:317-323

Graham FL, van der Eb AJ 1973 Transformation of rat cells by DNA of human adenovirus 5. *Virology* **54**:536-539

Grandchamp B, De Verneuil H, Beaumont C, Chretien S, Walter O, Nordmann Y 1987 Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. *Eur J Biochem* **162**:105-110

Greenwood AK, Butler PC, White RB, DeMarco U, Pearce D, Fernald RD 2003 Multiple corticosteroid receptors in a teleost fish: Distinct sequences, expression patterns, and transcriptional activities. *Endocrinology* (in press)

Griffin C, Flouriot G, Sonntag-Buck V, Nestor P, Gannon F 1998 Identification of novel chicken estrogen receptor-alpha messenger ribonucleic acid isoforms generated by alternative splicing and promoter usage. *Endocrinology* **139**:4614-4625

Gruol DJ, Rajah FM, Bourgeois S 1989 Cyclic AMP-dependent protein kinase modulation of the glucocorticoid-induced cytolytic response in murine T-lymphoma cells. *Mol Endocrinol* **3**:2119-2127

Hager GL, Fletcher TM, Xiao N, Baumann CT, Muller WG, McNally JG 2000 Dynamics of gene targeting and chromatin remodelling by nuclear receptors. *Biochem Soc Trans* **28**:405-410

Hammond GL 1988 Molecular analyses of human corticosteroid-binding globulin. Expression and gene structure. *Ann N Y Acad Sci* **538**:25-29

Hanson RW, Reshef L 1997 Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* **66**:581-611

Harris M 1984 High frequency induction by 5-azacytidine of proline independence in CHO-K1 cells. *Som Cell Mol Genet* **10**:615-624.

Harvey C, Jackson SM, Siddiqui SK, Gutierrez-Hartmann A 1991 Structure-function analysis of the rat prolactin promoter: phasing requirements of proximal cell-specific elements. *Mol Endocrinol* **5**:836-843

Hebbar PB, Archer TK 2003 Chromatin remodeling by nuclear receptors. *Chromosoma* **111**:495-504

Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson J, Bronnegard M, Wikstrom AC 1997 Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. *J Biol Chem* **272**:26659-26664

Herman JP, Spencer R 1998 Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. *J Neurosci* **18**:7462-7473

Herman JP, Patel PD, Akil H, Watson SJ 1989 Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. *Mol Endocrinol* **3**:1886-1894

Hoeck W, Rusconi S, Groner B 1989 Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. *J Biol Chem* **264**:14396-14402

Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318**:635-641

Hollenberg SM, Evans RM 1988 Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell* **55**:899-906

Holmes MC, French KL, Seckl JR 1997 Dysregulation of diurnal rhythms of serotonin 5-HT2C and corticosteroid receptor gene expression in the hippocampus with food restriction and glucocorticoids. *J Neurosci* **17**:4056-4065

Honda M, Orii F, Ayabe T, Imai S, Ashida T, Obara T, Kohgo Y 2000 Expression of glucocorticoid receptor beta in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. *Gastroenterology* **118**:859-866

Hsu DT, Chen FL, Takahashi LK, Kalin NH 1998 Rapid stress-induced elevations in corticotropin-releasing hormone mRNA in rat central amygdala nucleus and hypothalamic paraventricular nucleus: an in situ hybridization analysis. *Brain Res* **788**:305-310

Hurley DM, Accili D, Stratakis CA, Karl M, Vamvakopoulos N, Rorer E, Constantine K, Taylor SI, Chrousos GP 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. *J Clin Invest* **87**:680-686

Jaffe HL 1924 The influence of the suprarenal gland on the thymus. *J Exp Med XL*, 325-343.

Jondal M, Pazirandeh A, Okret S 2001 A role for glucocorticoids in the thymus? *Trends Immunol* **22**:185-186

Kalinyak JE, Dorin RI, Hoffman AR, Perlman AJ 1987 Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. *J Biol Chem* **262**:10441-10444

Karin M 1998 New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? *Cell* **93**:487-490

Kass SU, Pruss D, Wolffe AP 1997 How does DNA methylation repress transcription? *Trends Genet* **13**:444-449

Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P 1990 Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* **9**:1603-1614

Keightley MC, Curtis AJ, Chu S, Fuller PJ 1998 Structural determinants of cortisol resistance in the guinea pig glucocorticoid receptor. *Endocrinology* **139**:2479-2485

Kellendonk C, Tronche F, Reichardt HM, Schutz G 1999 Mutagenesis of the glucocorticoid receptor in mice. *J Steroid Biochem Mol Biol* **69**:253-259

Keshet I, Lieman-Hurwitz J, Cedar H 1986 DNA methylation affects the formation of active chromatin. *Cell* **44**:535-543

Kitraki E, Karandrea D, Kittas C 1999 Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain. *Neuroendocrinology* **69**:331-338

Korn SH, Koerts-de Lang E, Engel GE, Arends JW, Wouters EF, Thunnissen FB 1998 Alpha and beta glucocorticoid receptor mRNA expression in skeletal muscle. *J Muscle Res Cell Motil* **19**:757-765

Kornberg RD 1977 Structure of chromatin. *Annu Rev Biochem* **46**:931-954

Kos M, Reid G, Denger S, Gannon F 2001 Minireview: genomic organization of the human ERalpha gene promoter region. *Mol Endocrinol* **15**:2057-2063

Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CR, Seckl JR, Mullins JJ 1997 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci U S A* **94**:14924-14929

Kozak M 1991 An analysis of vertebrate mRNA sequences: intimations of translational control. *J Cell Biol* **115**:887-903

Kwak SP, Patel PD, Thompson RC, Akil H, Watson SJ 1993 5'-Heterogeneity of the mineralocorticoid receptor messenger ribonucleic acid: differential expression and regulation of splice variants within the rat hippocampus. *Endocrinology* **133**:2344-2350

Lai M, McCormick JA, Chapman KE, Kelly PA, Seckl JR, Yau JL 2003 Differential regulation of corticosteroid receptors by monoamine neurotransmitters and antidepressant drugs in primary hippocampal culture. *Neuroscience* **118**:975-984

Leclerc S, Palaniswami R, Xie BX, Govindan MV 1991a Molecular cloning and characterization of a factor that binds the human glucocorticoid receptor gene and represses its expression. *J Biol Chem* **266**:17333-17340

Leclerc S, Xie BX, Roy R, Govindan MV 1991b Purification of a human glucocorticoid receptor gene promoter-binding protein. Production of polyclonal antibodies against the purified factor. *J Biol Chem* **266**:8711-8719

Leroy P, Krust A, Zelent A, Mendelsohn C, Garnier JM, Kastner P, Dierich A, Chambon P 1991 Multiple isoforms of the mouse retinoic acid receptor alpha are generated by alternative splicing and differential induction by retinoic acid. *EMBO J* **10**:59-69

Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, Chrousos GP, Klemm DJ 1997 Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med* **186**:1567-1574

Levi A, Shechter Y, Neufeld EJ, Schlessinger J 1980 Mobility, clustering, and transport of nerve growth factor in embryonal sensory cells and in a sympathetic neuronal cell line. *Proc Natl Acad Sci U S A* **77**:3469-3473

Listwak SJ, Gold PW, Whitfield HJ, Jr. 1996 The human mineralocorticoid receptor gene promoter: its structure and expression. *J Steroid Biochem Mol Biol* **58**:495-506

Liu D, Diorio J, Day JC, Francis DD, Meaney MJ 2000 Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat Neurosci* **3**:799-806

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**:497-505

Lupien SJ, McEwen BS 1997 The acute effects of corticosteroids on cognition: integration of animal and human model studies. *Brain Res Brain Res Rev* **24**:1-27

Mallampalli RK, Walter ME, Peterson MW, Hunninghake GW 1994 Betamethasone activation of CTP:cholinephosphate cytidylyltransferase in vivo is lipid dependent. *Am J Respir Cell Mol Biol* **10**:48-57

Malkoski SP, Dorin RI 1999 Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. *Mol Endocrinol* **13**:1629-1644

Maruvada P, Baumann CT, Hager GL, Yen PM 2003 Dynamic shuttling and intranuclear mobility of nuclear hormone receptors. *J Biol Chem* **278**:12425-12432

McCormick JA. 2000 Transcriptional Regulation of the Rat Glucocorticoid Receptor Gene. *PhD Thesis*. University of Edinburgh

McCormick JA, Lyons V, Jacobson MD, Noble J, Diorio J, Nyirenda M, Weaver S, Ester W, Yau JL, Meaney MJ, Seckl JR, Chapman KE 2000 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Mol Endocrinol* **14**:506-517

McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, Weiss JM 1997 The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res Brain Res Rev* **23**:79-133

McEwen BS 2000 The neurobiology of stress: from serendipity to clinical relevance. *Brain Res* **886**:172-189

McIntyre WR, Samuels HH 1985 Triamcinolone acetonide regulates glucocorticoid-receptor levels by decreasing the half-life of the activated nuclear-receptor form. *J Biol Chem* **260**:418-427

McKay LI, Cidlowski JA 1999 Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev* **20**:435-459

McNally JG, Muller WG, Walker D, Wolford R, Hager GL 2000 The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* **287**:1262-1265

Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM 1988 Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* **239**:766-768

Meaney MJ, Diorio J, Francis D, Weaver S, Yau J, Chapman K, Seckl JR 2000 Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. *J Neurosci* **20**:3926-3935

Miesfeld R, Rusconi S, Godowski PJ, Maler BA, Okret S, Wikstrom AC, Gustafsson JA, Yamamoto KR 1986 Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. *Cell* **46**:389-399

Miller AH, Spencer RL, Pearce BD, Pisell TL, Azrieli Y, Tanapat P, Moday H, Rhee R, McEwen BS 1998 Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. *Cell Immunol* **186**:45-54

Mitchell JB, Betito K, Rowe W, Boksa P, Meaney MJ 1992 Serotonergic regulation of type II corticosteroid receptor binding in hippocampal cell cultures: evidence for the importance of serotonin-induced changes in cAMP levels. *Neuroscience* **48**:631-639

Morris DR, Geballe AP 2000 Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol* **20**:8635-8642

Ng HH, Bird A 1999 DNA methylation and chromatin modification. *Curr Opin Genet Dev* **9**:158-163

Nobukuni Y, Smith CL, Hager GL, Detera-Wadleigh SD 1995 Characterization of the human glucocorticoid receptor promoter. *Biochemistry* **34**:8207-8214

Nunez BS, Vedeckis WV 2002 Characterization of promoter 1B in the human glucocorticoid receptor gene. *Mol Cell Endocrinol* **189**:191-199

Nyirenda MJ, Lindsay RS, Kenyon CJ, Burchell A, Seckl JR 1998 Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *J Clin Invest* **101**:2174-2181

Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA 1999 The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem* **274**:27857-27866

Oakley RH, Sar M, Cidlowski JA 1996 The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *J Biol Chem* **271**:9550-9559

Oakley RH, Webster JC, Sar M, Parker CR, Jr., Cidlowski JA 1997 Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. *Endocrinology* **138**:5028-5038

Odermatt A, Arnold P, Frey FJ 2001 The intracellular localization of the mineralocorticoid receptor is regulated by 11beta-hydroxysteroid dehydrogenase type 2. *J Biol Chem* **276**:28484-28492

Okret S, Poellinger L, Dong Y, Gustafsson JA 1986 Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. *Proc Natl Acad Sci U S A* **83**:5899-5903

Olsson T, Mohammed AH, Donaldson LF, Henriksson BG, Seckl JR 1994 Glucocorticoid receptor and NGFI-A gene expression are induced in the hippocampus after environmental enrichment in adult rats. *Brain Res Mol Brain Res* **23**:349-353

Orth DN, Kovacs WJ, DeBold CR. 1992 The Adrenal Cortex. Williams Textbook of Endocrinology. [8th Edition], pp489-619. Edited by Wilson JD & Foster DW.

Orti E, Mendel DB, Smith LI, Munck A 1989 Agonist-dependent phosphorylation and nuclear dephosphorylation of glucocorticoid receptors in intact cells. *J Biol Chem* **264**:9728-9731

Otto C, Reichardt HM, Schutz G 1997 Absence of glucocorticoid receptor-beta in mice. *J Biol Chem* **272**:26665-26668

Palkovits M, Young WS, III, Kovacs K, Toth Z, Makara GB 1998 Alterations in corticotropin-releasing hormone gene expression of central amygdaloid neurons following long-term paraventricular lesions and adrenalectomy. *Neuroscience* **85**:135-147

Pardridge WM 1987 Plasma protein-mediated transport of steroid and thyroid hormones. *Am J Physiol* **252**(2 Pt 1):E157-164.

Pazirandeh A, Xue Y, Prestegaard T, Jondal M, Okret S 2002 Effects of altered glucocorticoid sensitivity in the T-cell lineage on thymocyte and T-cell homeostasis. *FASEB J* **16**(7):727-729

Pearce D, Yamamoto KR 1993 Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. *Science* **259**:1161-1165

Pepin M-C, Pothier F, Barden N 1995 Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. *Nature* **355**:725-728

Picard D, Yamamoto KR 1987 Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J* **6**:3333-3340

Pitot HC, Peraino C, Morse Jr. PA, Potter VR. 1964 Hepatomas in tissue culture compared with adapting liver *in vivo*. *National Cancer Institutue Monograph* **13**:229-245

Ponger L, Duret L, Mouchiroud D 2001 Determinants of CpG islands: expression in early embryo and isochore structure. *Genome Res* **11**:1854-1860

Pratt WB, Toft DO 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* **18**:306-360

Preiss T, Hentze MW 1999 From factors to mechanisms: translation and translational control in eukaryotes. *Curr Opin Genet Dev* **9**:515-521

Purton JF, Boyd RL, Cole TJ, Godfrey DI 2000 Intrathymic T cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling. *Immunity* **13**:179-186

Raber J 1998 Detrimental effects of chronic hypothalamic-pituitary-adrenal axis activation. From obesity to memory deficits. *Mol Neurobiol* **18**:1-22

Rajput B, Shaper NL, Shaper JH 1996 Transcriptional regulation of murine beta1,4-galactosyltransferase in somatic cells. Analysis of a gene that serves both a housekeeping and a mammary gland-specific function. *J Biol Chem* **271**:5131-5142

Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schutz G 1998 DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* **93**:531-541

Reichardt HM, Umland T, Bauer A, Kretz O, Schutz G 2000 Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. *Mol Cell Biol* **20**:9009-9017

Reik A, Schutz G, Stewart AF 1991 Glucocorticoids are required for establishment and maintenance of an alteration in chromatin structure: induction leads to a reversible disruption of nucleosomes over an enhancer. *EMBO J* **10**:2569-2576

Reynolds R. 2002 Programming of the hypothalamic-pituitary-adrenal axis during foetal life *PhD Thesis.* University of Edinburgh

Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB 2002 Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol Chem* **277**:5209-5218

Robyr D, Wolffe AP, Wahli W 2000 Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol Endocrinol* **14**:329-347

Rosewicz S, McDonald AR, Maddux BA, Goldfine ID, Miesfeld RL, Logsdon CD 1988 Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. *J Biol Chem* **263**:2581-2584

Sackey FN, Watson CS, Gametchu B 1997 Cell cycle regulation of membrane glucocorticoid receptor in CCRF-CEM human ALL cells: correlation to apoptosis. *Am J Physiol* **273**(3 Pt 1):E571-83.

Sakai H, Toyota N, Ito F, Takahashi H, Hashimoto Y, Iizuka H 1999 Glucocorticoids inhibit proliferation and adhesion of the IL-3-dependent mast cell line, MC/9, to NIH/3T3 fibroblasts, with an accompanying decrease in IL-3 receptor expression. *Arch Dermatol Res* **291**:224-231

Sambrook J, Fritsch EF, Maniatis 2001 T. Molecular Cloning; A Laboratory Manual. [3rd Edition] *Cold Spring Harbour Laboratory Press.*

Sapolsky RM, Krey LC, McEwen BS 1984 Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology* **114**:287-292

Schaaf MJ, Cidlowski JA 2003 Molecular determinants of glucocorticoid receptor mobility in living cells: the importance of ligand affinity. *Mol Cell Biol* **23**:1922-1934

Schibler U, Hagenbuchle O, Wellauer PK, Pittet AC 1983 Two promoters of different strengths control the transcription of the mouse alpha-amylase gene Amy-1a in the parotid gland and the liver. *Cell* **33**:501-508

Schubert D, Heinemann S, Carlisle W, Tarikas H, Kimes B, Patrick J, Steinbach JH, Culp W, Brandt BL 1974 Clonal cell lines from the rat central nervous system. *Nature* **249**:224-227

Schmid W, Strahle U, Schutz G, Schmitt J, Stunnenberg H 1989 Glucocorticoid receptor binds cooperatively to adjacent recognition sites. *EMBO J* **8**:2257-2263

Seckl JR 1997 Glucocorticoids, feto-placental 11 beta-hydroxysteroid dehydrogenase type 2, and the early life origins of adult disease. *Steroids* **62**:89-94

Seckl JR 2000 Antenatal glucocorticoid therapy: a caveat to the applause. *Clin Sci (Colch)* **98**:127-128

Seckl JR, Chapman KE 1997 Medical and physiological aspects of the 11beta-hydroxysteroid dehydrogenase system. *Eur J Biochem* **249**:361-364

Seckl JR, Nyirenda MJ, Walker BR, Chapman KE 1999 Glucocorticoids and fetal programming. *Biochem Soc Trans* **27**:74-78

Seckl JR, Olsson T 1995 Glucocorticoid hypersecretion and the age-impaired hippocampus: cause or effect? *J Endocrinol* **145**:201-211

Seckl JR, Yau J, Holmes M 2002 11Beta-hydroxysteroid dehydrogenases: a novel control of glucocorticoid action in the brain. *Endocr Res* **28**:701-707

Selvakumar M, Helfman DM 1999 Exonic splicing enhancers contribute to the use of both 3' and 5' splice site usage of rat beta-tropomyosin pre-mRNA. *RNA* **5**:378-394

Shi YB, Yaoita Y, Brown DD 1992 Genomic organization and alternative promoter usage of the two thyroid hormone receptor beta genes in Xenopus laevis. *J Biol Chem* **267**:733-738

Singer CJ, Khan MS, Rosner W 1988 Characteristics of the binding of corticosteroid-binding globulin to rat cell membranes. *Endocrinology* **122**:89-96

Singh H, Sen R, Baltimore D, Sharp PA 1986 A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature* **319**:154-158

Stapleton C, Somma MP, Lavin P 1993 Cell type-specific interactions of transcription factors of transcription factors with a housekeeping promoter in vivo. *Nucleic Acids Res* **21**:2465-2471.

Steinberg RA, O'Farrell PH, Friedrich U, Coffino P 1977 Mutations causing charge alterations in regulatory subunits of the cAMP-dependent protein kinase of cultured S49 lymphoma cells. *Cell* **10**:381-391

Stewart PM, Whorwood CB, Mason JI 1995 Type 2 11 beta-hydroxysteroid dehydrogenase in foetal and adult life. *J Steroid Biochem Mol Biol* **55**:465-471

Strahle U, Schmid W, Schutz G 1988 Synergistic action of the glucocorticoid receptor with transcription factors. *EMBO J* **7**:3389-3395

Strahle U, Schmidt A, Kelsey G, Stewart AF, Cole TJ, Schmid W, Schutz G 1992 At least three promoters direct expression of the mouse glucocorticoid receptor gene. *Proc Natl Acad Sci U S A* **89**:6731-6735

Suzuki N, Del Villar K, Tamanoi F 1998 Farnesyltransferase inhibitors induce dramatic morphological changes of KNRK cells that are blocked by microtubule interfering agents. *Proc Natl Acad Sci U S A* **95**:10499-10504

Tazi J, Bird A 1990 Alternative chromatin structure at CpG islands. *Cell* **60**:909-920

Thomassin H, Flavin M, Espinas ML, Grange T 2001 Glucocorticoid-induced DNA demethylation and gene memory during development. *EMBO J* **20**:1974-1983

Tian H, Kole R 2001 Strong RNA splicing enhancers identified by a modified method of cycled selection interact with SR protein. *J Biol Chem* **276**:33833-33839

Tronche F, Kellendonk C, Reichardt HM, Schutz G 1998 Genetic dissection of glucocorticoid receptor function in mice. *Curr Opin Genet Dev* **8**:532-538

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G 1999 Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* **23**:99-103

van der Velden AW, Thomas AA 1999 The role of the 5' untranslated region of an mRNA in translation regulation during development. *Int J Biochem Cell Biol* **31**:87-106

Vanderbilt JN, Miesfeld R, Maler BA, Yamamoto KR. 1987 Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. *Mol Endocrinol* **1**:68-74.

Vazquez DM, Lopez JF, Morano MI, Kwak SP, Watson SJ, Akil H 1998 Alpha, beta, and gamma mineralocorticoid receptor messenger ribonucleic acid splice variants: differential expression and rapid regulation in the developing hippocampus. *Endocrinology* **139**:3165-3177

Voice MW, Seckl JR, Edwards CR, Chapman KE 1996 11 beta-hydroxysteroid dehydrogenase type 1 expression in 2S FAZA hepatoma cells is hormonally regulated: a model system for the study of hepatic glucocorticoid metabolism. *Biochem J* **317**:621-625

Warriar N, Page N, Govindan MV 1996 Expression of human glucocorticoid receptor gene and interaction of nuclear proteins with the transcriptional control element. *J Biol Chem* **271**:18662-18671

Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M, Cidlowski JA 1997 Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. *J Biol Chem* **272**:9287-9293

Webster JC, Oakley RH, Jewell CM, Cidlowski JA 2001 Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. *Proc Natl Acad Sci U S A* **98**:6865-6870

Wehling M 1997 Specific, nongenomic actions of steroid hormones. *Annu Rev Physiol* **59**:365-393

Weintraub H, Groudine M. 1976 Transcriptionally active and inactive conformations of chromosomal subunits. *Science* **193**:848-856.

Welberg LA, Seckl JR 2001 Prenatal stress, glucocorticoids and the programming of the brain. *J Neuroendocrinol* **13**:113-128

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M 1990 Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**:1205-1215

Yudt MR, Cidlowski JA 2001 Molecular identification and characterization of a and b forms of the glucocorticoid receptor. *Mol Endocrinol* **15**:1093-1103

Yudt MR, Cidlowski JA 2002 The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. *Mol Endocrinol* **16**:1719-1726

Zelent A, Mendelsohn C, Kastner P, Krust A, Garnier JM, Ruffenach F, Leroy P, Chambon P 1991 Differentially expressed isoforms of the mouse retinoic acid receptor beta generated by usage of two promoters and alternative splicing. *EMBO J* **10**:71-81

Zennaro MC, Keightley MC, Kotelevtsev Y, Conway GS, Soubrier F, Fuller PJ 1995 Human mineralocorticoid receptor genomic structure and identification of expressed isoforms. *J Biol Chem* **270**:21016-21020

Zennaro MC, Le Menuet D, Lombes M 1996 Characterization of the human mineralocorticoid receptor gene 5'-regulatory region: evidence for differential hormonal regulation of two alternative promoters via nonclassical mechanisms. *Mol Endocrinol* **10**:1549-1560

Zhang J, Byrne CD. 2000 Differential hepatic lobar gene expression in offspring exposed to altered maternal dietary protein intake. *Am J Physiol Gatstrointest Liver Physiol* **278**:128-136.

Zong J, Ashraf J, Thompson EB 1990 The promoter and first, untranslated exon of the human glucocorticoid receptor gene are GC rich but lack consensus glucocorticoid receptor element sites. *Mol Cell Biol* **10**:5580-5585